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T Follicular Helper Cells in Health and Inflammatory Bowel Disease

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**T Follicular Helper Cells in Health and
Inflammatory Bowel Disease**

By

Jessica Elanor Thomas

A thesis submitted for the degree of Doctor of
Philosophy

2013

Abstract

The intestinal IgA response has features that are different to those of the systemic humoral response, which is dominated by IgG. Although the IgA response, like the IgG response, includes an antigen specific component, it is also associated with polyspecificity and autoimmunity. The profile of intestinal immunoglobulins changes in inflammatory bowel disease (IBD) where there is a disproportionate increase in IgG production and in ulcerative colitis (UC), this includes the production of autoantibodies. In this thesis, two immunoregulatory T cell subsets that could influence the intestinal B cell response have been studied; T follicular helper cells (T_{FH}) and regulatory T cells (T_{reg}).

Results in chapter 3 show that there is a higher density of T_{FH} in gut associated lymphoid tissue (GALT) compared to peripheral lymphoid tissue due to a higher density of CD57- T_{FH} . The expression of cytokines and CD40L was almost comparable between CD57+ and CD57- T_{FH} . However, culturing experiments suggest that CD57- T_{FH} may develop into CD57+ T_{FH} and there is a constant turnover of T_{FH} in the gut.

Experiments in chapter 4 attempted to seek evidence for a developmental relationship between T_{FH} and T_{reg} by analysis of T cell receptor sequences. No evidence of plasticity between these subsets was observed.

Experiments in chapter 5 set out to characterise T_{FH} in IBD. In the appendix of UC patients, nearly all T_{FH} were CD57+ and at a high density within germinal centres (GC).

This thesis concludes that T_{FH} are more phenotypically diverse and denser in GALT compared to peripheral lymphoid tissue. This may reduce the threshold for GC B cell survival in the gut permitting the propagation of plasma cells that secrete polyspecific

and autoreactive IgA. T_{FH} are denser still in the small GC in UC appendix. This may be relevant to the production of autoantibodies and disease pathogenesis.

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Abbreviations

AID- Activation-induced cytidine deaminase

APC- Antigen presenting cells

APRIL- A proliferation inducing ligand

BCR- B cell receptor

CD- Crohns Disease

CD40L- CD40 ligand

CSR- Class switch recombination

DC- Dendritic cell

DNA-PK - DNA-dependent protein kinase

DN-Double negative for CD4 and CD8

FACS- Fluorescent activated cell sorting

Foxp3- Forkhead box P3

GALT- Gut associated lymphoid tissue

GC- Germinal centre

GWAS - Genome wide association studies

HBI- Harvey Bradshaw index

HEL- Hen egg lysozyme

IBD- Inflammatory Bowel Disease

ICOS- Inducible co-stimulator

IFN- γ - Interferon gamma

Ig- Immunoglobulin

IHC- Immunohistochemistry

IL- Interleukin

ILF-Isolated lymphoid follicle

IPEX- Immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome

iTreg- Induced regulatory T cell

K/O-Knock out

LN- Lymph nodes

LP- Lamina propria

LTi cells- Lymphoid tissue inducer cells.

MadCAM- Mucosal addressin cell adhesion molecule-1

MALT- Mucosa associated lymphoid tissues

MHC- Major histocompatibility complex

MSMD -Mendelian susceptibility to mycobacterial disease

NALT- Nasal-associated lymphoid tissue

NFAT - Nuclear factor of activated T cells

NHEJ- Non homologous end joining

NK cells- Natural killer cells

nTreg- Natural regulatory T cell

PCR- Polymerase chain reaction

PD-1 -Programmed death 1

PDE3B- Cyclic nucleotide phosphodiesterase 3B

PD-L1 –Programmed death ligand 1

PD-L2– Programmed death ligand 2

PP- Peyer's patch

Pre-B cell- Precursor B cell

Pro-B cell- Progenitor B cell

RAG- Recombination activating gene

RALDH- Retinaldehyde dehydrogenase

RSS - Recombination signal sequences

RT-PCR- Real-time polymerase chain reaction

SHM- Somatic hyper mutation

SCCAI - Simple Colitis Clinical Activity index

SNP- Single nucleotide polymorphism

SP- Single positive

Tconv- Conventional T cells

TCR- T cell receptor

TD- T cell dependent

TdT- Terminal deoxynucleotidyl transferase

T_{FH}- T follicular helper cells

Tfr- T follicular regulatory cells

TGF- β - Transforming growth factor- β

TI- T cell independent

Treg- Regulatory T cells

UC- Ulcerative colitis

V(D)J- Variable-diversity-joining

XSCID- X-linked severe combined immunodeficiency

1 Chapter 1 Introduction

This thesis will describe investigations of two different immunoregulatory T cell subsets in the gut and peripheral lymphoid tissues. Three chapters of experimental data will investigate possible differences in T follicular helper cell (T_{FH}) populations in the gut compared to peripheral lymphoid tissue, the possibility of clonal relationships between T_{FH} and regulatory T cells (Treg) and changes in these T cell subset populations that occur in inflammatory bowel disease (IBD).

To introduce this data, T_{FH} and Treg will be described in the context of T cell development, function and interaction with B cells. The function of gut associated lymphoid tissue (GALT) will be discussed to consider how differences in B cells generated in GALT compared to the peripheral lymphoid tissue might be modulated by differences in immunoregulatory T cells. Features of IBD will be discussed to consider how aberrant function of immunoregulatory T cells might contribute to the disease pathogenesis.

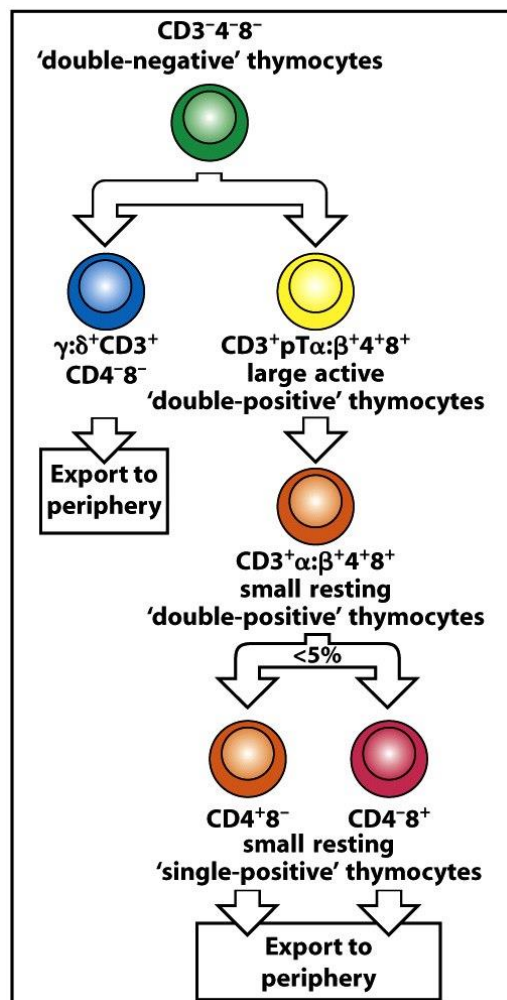
1.1 T cell development

T cells develop in the thymus from bone marrow derived pluripotent progenitors that have the ability to give rise to T cells, B cells or natural killer (NK) cells (Kondo et al., 1997). The importance of the thymus for T cell development was originally demonstrated by the development of immunodeficiency in mice that had undergone a neonatal thymectomy (Miller, 2002, Miller, 1961).

When haematopoietic progenitors arrive in the thymus they receive signalling through Notch that modifies gene expression and commits the cell to a T cell lineage (Pui et al., 1999, Radtke et al., 1999, Hayday and Pennington, 2007, Naito et al., 2011). Two distinct lineages of T cells develop in the thymus that differs in their T cell receptor (TCR). They express either an $\alpha\beta$ TCR or a $\gamma\delta$ TCR. The $\alpha\beta$ T cells can be

further sub divided by their surface expression of either CD4 or CD8 and the choice between CD4 and CD8 expression is also made within the thymus (Germain, 2002).

The stages of T cell development within the thymus can be defined by the expression of CD4 and CD8. The thymocytes begin development double negative for CD4 and CD8 (DN) and during the DN stage the TCR genes, except for α , rearrange. Commitment to either an $\alpha\beta$ or $\gamma\delta$ lineage is made just before the end of the DN stage (Fig 1.1) (Ciofani et al., 2006, Ciofani and Zúñiga-Pflücker, 2010, Hayday and Pennington, 2007).



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Figure 1-1 Stages of T cell development can be distinguished by the expression of CD4 and CD8.

1.1.1 *TCR rearrangement*

T cell diversity and specificity for peptides arises from the genetic rearrangement of the genes encoding the TCR (fig 1.2). There are four different genes encoding the TCR: *Tcra*, *Tcrb*, *Tcrg* and *Tcrd*. Diversity is generated by recombination of variable (V), diversity (D) and joining (J) gene segments at the *tcr* loci (Krangel, 2009). The TCR chains produced from these genes are expressed on the cell surface as a pair, as either an $\alpha\beta$ TCR or a $\gamma\delta$ TCR. The TCR β and δ chains are produced from V, D and J segments whereas the α and γ chains are made up of only V and J segments (Bassing et al., 2002). There are several V, (D) and J segments within each locus and joining these segments in different combinations produces combinatorial diversity of the TCR.

Within the thymus, thymocytes express RAG-1 and RAG-2 which make up the RAG recombinase (Oettinger et al., 1990). RAG proteins recognise specific sites called recombination signal sequences (RSSs) at V, (D) and J segments within the *tcr* loci (Bassing et al., 2002, Krangel, 2009, Santagata et al., 1999). The RSS consist of a conserved heptamer and an AT rich nonamer separated by a 12 or 23 bp spacer (Grawunder et al., 1998). A segment with a RSS containing a 12 bp spacer can only join with a segment with a RSS containing 23bp spacer ensuring that segments join together in the correct order of VDJ or VJ (Bassing et al., 2002).

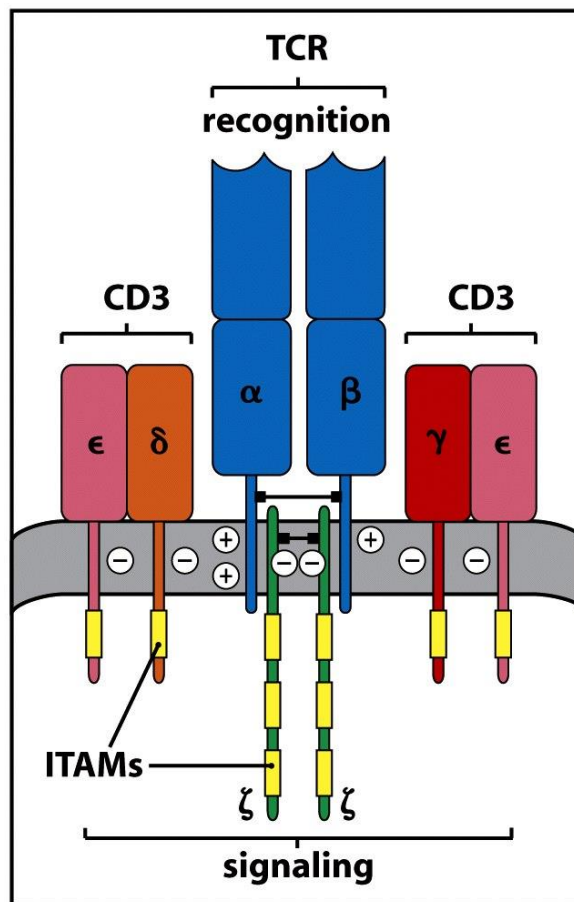
V, (D) J recombination begins when the RAG proteins, which have endonuclease activity, makes nicks at the RSS of the two segments to be joined (van Gent et al., 1996). RAG catalyses the formation of a hair pin loop where the 3' end of the break invades the opposite strand (Gent et al., 1996). The coding region ends are held together by Ku70:Ku80 heterodimeric complex and the hairpins are opened up by a complex of Artemis and DNA-dependent protein kinase (DNA-PK) (Gu et al., 1997, Moshous et al., 2001, Ma et al., 2002, Gellert, 2002). DNA repair enzymes remove nucleotides at

the open ends while terminal deoxynucleotidyl transferase (TdT) adds nucleotides before the ends are joined together by DNA ligase IV and Xrcc4 (Ma et al., 2002, Benedict et al., 2000, Gellert, 2002, Murphy et al., 2008). This results in imprecise joining of the coding regions between V, D and J segments, which adds another level of diversity between TCRs and creates a unique sequence which is carried by the T cell and its clonal progeny. When two RSSs are joined together the region in between is excised and precisely joined together creating an excision circle (Bassing et al., 2002). If the sequence generated by V(D)J recombination is out of frame or non-coding, recombination can occur on the second allele (Bassing et al., 2002).

1.1.2 *Lineage commitment*

During the double negative thymocyte stage, the TCR δ , TCR γ and TCR β rearrange (Blom et al., 1999, Hayday and Pennington, 2007). After successful rearrangement of these TCR loci the thymocytes either express a pre-TCR or $\gamma\delta$ TCR on the cell surface (Ciofani and Zúñiga-Pflücker, 2010). The pre-TCR is made up of the rearranged TCR β chain and a pre-TCR alpha chain, as the TCR α chain has yet to undergo recombination. At this stage there is a checkpoint and if signalling occurs through the pre-TCR or $\gamma\delta$ TCR expressed on the cell surface, then the thymocyte continues in development (Ciofani and Zúñiga-Pflücker, 2010). Though the γ and δ chains are thought to rearrange before the β chain, the majority of thymocytes commit to an $\alpha\beta$ lineage (Livák et al., 1999). The exact mechanism for commitment to either a $\gamma\delta$ or $\alpha\beta$ lineage is not known. However one model suggests that the strength of signal through the pre-TCR and $\gamma\delta$ TCR determines the cells fate (Ciofani and Zúñiga-Pflücker, 2010). From this point the $\gamma\delta$ thymocytes continue on a separate developmental path from $\alpha\beta$ thymocytes and the $\gamma\delta$ thymocytes do not revise their TCR any further (Prinz et al., 2006). The $\gamma\delta$ T cells mostly remain CD4-CD8- double negative (Ciofani and Zúñiga-Pflücker, 2010). The thymocytes committed to an $\alpha\beta$ lineage continue their development by rearranging

the α chain and become CD4+CD8+ double positive (DP) (Ciofani and Zúñiga-Pflücker, 2010).



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Figure 1-2 The $\alpha\beta$ T cell receptor complex.

The TCR complex as a whole is needed for efficient signalling. The TCR α and β chains are expressed on the cell surface as a TCR heterodimer. The TCR complex is associated with a homodimer of ζ chains. The CD3 complex consists of heterodimers of CD3 ϵ and δ chains and γ and ϵ chains. Both ζ chains and CD3 chains contain ITAM signalling motifs (Murphy et al., 2008).

1.1.3 Using TCR chain rearrangements to detect clonally related T cells

1.1.3.1 Detection of T cell clones by analysis of TCR γ chain rearrangements

As described above, recombination of the TCR β , γ and δ chains occurs before thymocytes commit to either a $\gamma\delta$ or $\alpha\beta$ T cell lineage. Therefore virtually all T cells of both $\gamma\delta$ or $\alpha\beta$ T cell lineages have undergone rearrangement of TCR δ and TCR γ in their

history. The rearranged TCR δ locus is located within the TCR α locus and is therefore deleted when the TCR α subsequently rearranges in $\alpha\beta$ T cells. However, the rearranged TCR γ chain is retained. The *tcr γ* locus has relatively few V and J exons compared to the other TCR genes and only one junctional region is formed where the V and J segments join. By detecting TCR γ rearrangements in T cells through PCR and sequencing the product, the junctional region can be analysed. Cells with the same V and J segments and junctional regions can be assumed to be clonally related. Thus the sequence of rearranged *tcr γ* locus can serve as a signature of clone of both $\gamma\delta$ or $\alpha\beta$ T cell lineages. This method has been used diagnostically for the detection of T cell malignancy and T cell clonality (McCarthy et al., 1991, McCarthy et al., 1992, Diss et al., 1995, Golby et al., 1999).

1.1.3.2 Detection of T cell clones in large and diverse polyclonal populations.

Rearrangements of the TCR β chain can also be used to identify T cell clonality (McCarthy et al., 1991, Dogan et al., 1996). However, whether either TCR β or TCR γ are targeted, it can be difficult to detect a minor population of clonally related cells in a large and diverse polyclonal background because the sequenced PCR products following amplification may be all different and the probability of detecting two members of a clone is low.

One method to detect members of a T cell clone in polyclonal T cell background is to use PCR primers specific for a TCR gene rearrangement to selectively amplify the same TCR gene rearrangement. Clone specific primers designed to bind to the unique junctional region of the TCR γ chain gene rearrangements can be used to efficiently detect clonally related T cells. Using this method, Golby et al. (1999) detected clonally expanded T_{FH} within tonsil tissue.

Detection of minor subsets of clonally related B cells in a polyclonal background has been achieved by specifically targeting members of a clone that have rearranged one of the many potentially used variable region gene segments. Related B lineage cells were detected within the intestinal mucosa in this way (Boursier et al., 2005). Comparing the sequences within a small fraction of the repertoire gives a greater probability of detecting related clones. This method has been adapted to the study of T cell clonality in Chapter 4 of this thesis, where a fraction of the TCR β repertoire was analysed to increase the probability of detecting clonally related sequences.

1.2 Positive and negative selection

At the DP stage after the TCR α chain has successfully rearranged, low levels of the $\alpha\beta$ TCR are expressed on the thymocyte surface (Ohashi et al., 1990). The DP thymocytes undergo positive selection that is dependent on an inherent germline-encoded specificity of the TCR and CD4 or CD8 co-receptor for MHC and also the recognition of self-peptide MHC complexes expressed by the thymic stromal cells (Morris and Allen, 2012, Germain, 2002, Murphy et al., 2008).

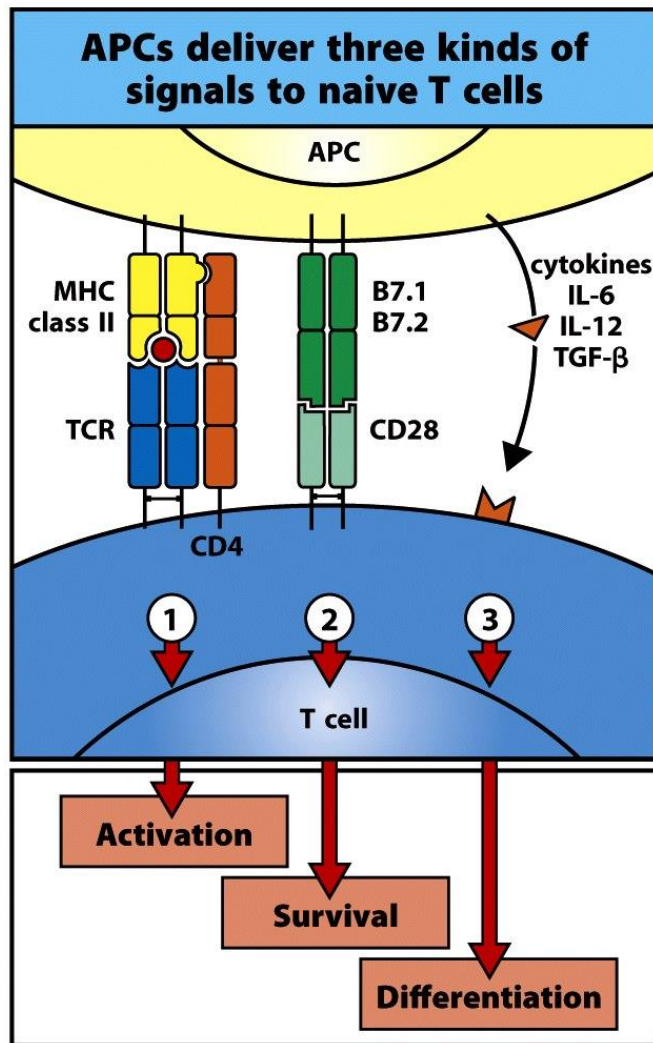
A cell that survives positive selection then expresses either CD4 or CD8 to become SP and commit to either class II or class I restriction respectively.

The developing thymocytes have highly variable TCR specificities and some of the thymocytes generated recognise self-peptides presented by thymic stromal cells. Negative selection ensures that thymocytes with TCR that have high affinity for self-peptides are clonally deleted by TCR mediated apoptosis. This mechanism limits the number of autoreactive T cells from entering the periphery and responsiveness to self-antigens (Palmer, 2003, Morris and Allen, 2012).

After passing positive and negative selection, 3 subsets of $\alpha\beta$ T cells are exported to the periphery: MHC class I restricted CD8 T cells, MHC class II restricted T cells and natural regulatory T cells (nTregs) that express the transcription factor forkhead box P3 (Foxp3) (Germain, 2002, Murphy et al., 2008).

1.3 T cell activation

The initial phase of a class II restricted immune response involves the processing and presentation of peptides by antigen presenting cells (APC) such as dendritic cells (DC). T cells with TCRs specific for the peptide become activated, proliferate and differentiate. This requires 3 separate signals from the APC (Kapsenberg, 2003). The first signal occurs through the TCR and co-receptor binding to the peptide:MHC complex on the APC (Kapsenberg, 2003). The second signal involves co-stimulatory signals such as the activation of CD28 on the surface of the T cell (Bour-Jordan and Bluestone, 2002). The third polarising signal promote differentiation into a certain T cell subset, depending on the pathogen, through modified cytokine secretion (Fig 1.3) (KaliDski et al., 1999).



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Figure 1-3 Activation of naive T cells by antigen presenting cells requires three signals.

T cell TCR binding a foreign antigen presented on MHC class II complex by the APC. 2- Co-stimulatory signal, for example CD28 on T cell binding to B7 molecules on the APC. 3- Differentiation signal by cytokines (Murphy, Travers et al. 2008).

1.4 T cell subsets

During an infection, activated CD4 T cells differentiate into different T cell subsets that secrete specific cytokines depending on the pathogen and the microenvironment in which the activation took place, to provide a tailored immune response. The specialist function of each T cell subset is controlled by transcriptional factors that direct the

cytokines produced and surface molecules expressed by the T cell (Murphy and Stockinger, 2010).

T cell activation results in the activation of important tyrosine kinases leading to the downstream activation of serine/threonine kinase proteins such as protein kinase C that subsequently activates the MAP kinase cascade and NF κ B. Also calcium signalling pathways are activated, such as the activation of calcineurin and consequently the dephosphorylation of nuclear factor of activated T cells (NFAT). Signalling culminates in the activation of three main groups of transcription factors: NFAT, NF κ B and AP1. NFAT and NF κ B translocate to the nucleus where they bind to gene targets and activate their transcription. The activation stimulus influences the genes that are targets and ultimately the type of T cell the cell will become. Master transcription factors are associated with each T cell subset and control the transcription of genes within the cell to polarise the cell to a particular phenotype (Murphy et al., 2008).

The first T cell subsets to be identified were Th1 and Th2 that were identified by the cytokines they produce (Mosmann and Coffman, 1989). Since the identification of Th1 and Th2 cells several other functional CD4 T cell subsets have been identified including inducible regulatory T cells (iTreg) Th17 and T follicular helper cells (T_{FH}) that are associated with expression of different transcription factors (Fig1.4).

Th1 cells express the transcription factor T-bet and produce IFN γ . The IFN γ produced by Th1 cells activates macrophages to kill intracellular pathogens (Murphy and Stockinger, 2010). IFN γ also promotes B cell immunoglobulin class switching to IgG which mediates opsonisation and phagocytosis of certain pathogens (Abbas et al., 1996).

Th2 cells express the transcription factor Gata3 and produce the cytokines, IL-4 and IL-5 (O'Shea and Paul, 2010, Abbas et al., 1996). Th2 cells response to helminth infections by activate eosinophils through IL-5 secretion and IL-4 promotes class switching to IgE (Abbas et al., 1996).

Th17 cells express the transcription factor Ror γ t and produce the cytokines IL-17a and IL-17f (O'Shea and Paul, 2010). Th17 cells recruit and intensify neutrophil activity in response to extracellular pathogens (Pelletier et al., 2010).

iTregs express the transcription factor foxp3 and produces cytokines such as IL-10 and TGF- β (O'Shea and Paul, 2010). iTregs suppress immune responses and will be discussed in more detail bellow.

T_{FH} express the transcription factor Bcl-6 and are associated with the production of the cytokine IL-21 (O'Shea and Paul, 2010). They provide help to B cells during affinity maturation to T cell dependent antigens (O'Shea and Paul, 2010). However there is still debate over whether T_{FH} are a distinct T cell subset or part of other T cell subsets as T_{FH} have the ability to produce Th1, Th2 and Th17 cytokines (Murphy and Stockinger, 2010).

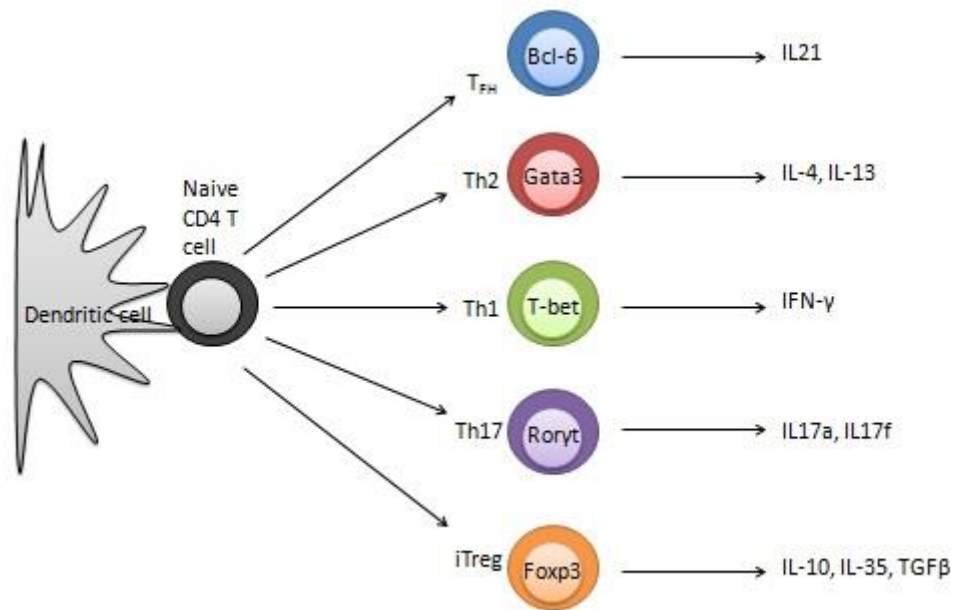


Figure 1-4 T cell subsets, master regulators and cytokines.

Adapted from O'Shea and Paul (2010).

1.5 Regulatory T cells

Regulatory cells are involved in controlling of immune responses and regulating immune homeostasis. Several different cell types with this ability have been identified including regulatory CD4 T cells, regulatory CD8 T cells and regulatory B cells. This thesis will concentrate on regulatory CD4 T cells (Tregs).

1.5.1 Regulatory T cell development

Treg were first identified by Sakaguchi et al. (1995) as a subset of CD4+CD25+ T cells which controlled immune tolerance. Depletion of this subset resulted in autoimmune disease (Sakaguchi et al., 1995).

There are two developmental pathways that generate Tregs. As discussed above, nTregs develop in the thymus during T cell development (Curotto de Lafaille and Lafaille, 2009). The exact events that lead to nTreg development over conventional

CD4 T cells (Tconv) development or clonal deletion are still unknown. Tregs have been shown to have self-reactive TCRs yet do not undergo negative selection possibly due to their TCR specificity, affinity, avidity and co-receptor signalling (Wirnsberger et al., 2011, Hsieh et al., 2004). nTreg are thought to have an avidity and affinity for self-antigens which is on the threshold between negative and positive selection (Wirnsberger et al., 2011).

During T cell development in the thymus, *foxp3* is upregulated in the cells fated to become nTregs and this normally occurs during the SP stage (Lee and Hsieh, 2009). *Foxp3* is both a repressor and activator of genes that contribute to Treg function and surface phenotype (Zheng et al., 2007). Maintained expression of *foxp3* is crucial for Treg development and function which is demonstrated in *foxp3* mutant scurfy mice that have a deficiency in Tregs and develop lethal autoimmune disease (Fontenot et al., 2003). *Foxp3* controls the transcription of a number of genes including promoting the transcription of genes encoding CTLA-4 and CD25 and the repression of *IL-7Ra*. *Foxp3* also represses cyclic nucleotide phosphodiesterase 3B (PDE3B) and down regulation of PDE3B allows the cell to adapt to chronic TCR and IL-2 signalling which are important features of Tregs (Gavin et al., 2007). Though functional *foxp3* is not essential for Treg survival, it is important in maintaining regulatory function (Gavin et al., 2007). **12.** Interestingly, in humans and not mice, a spliced variant of *foxp3* is expressed as well as the full length protein. The spliced variant mRNA lacks exon 2 but retains its ability to induce functional anergy but not to the same extent as the full length *foxp3* (Ziegler, 2006).

The second developmental pathway for Tregs occurs in the periphery and these Tregs are termed induced Tregs (iTreg). In vitro studies have demonstrated that iTregs can arise from Tconv by TCR signalling in the presence of IL-2 and TGF β which

drives the upregulation of foxp3 (Davidson et al., 2007). Analysis of the TCR repertoire between Tregs and Tconv suggests that only a small fraction of the Treg population is generated in the periphery and the majority of Tregs are generated in the thymus (Lathrop et al., 2008).

1.5.2 *Regulatory T cell function.*

Treg suppressive function is elicited through cell to cell contact and secreted molecules and involves CTLA-4, transforming growth factor- β (TGF- β) and IL-10.

CTLA-4 is constitutively expressed on Tregs. Mice with CTLA-4 knocked out specifically on Tregs developed systemic lymphocyte proliferation and fatal T cell-mediated autoimmune disease which demonstrates the importance of Treg CTLA-4 mediated interactions in controlling immune responses (Wing et al., 2008). Several mechanisms have been proposed to explain the regulatory effects of CTLA-4 including competing with CD28, down regulating CD80 and CD86, regulating signalling components and inhibiting lipid raft and microcluster formation needed for efficient signalling (Rudd, 2008, Wing et al., 2008). Rudd (2008) proposed that the cell intrinsic inhibitory function of CTLA-4 is through a “reverse stop-signal” mechanism. T cells need reduced motility to form long interactions with APC and reduced motility is initiated through TCR ligation. It is thought that CTLA-4 is able to reverse the stop signal that reduced T cell motility and therefore prevents stable immunological synapse formation (Rudd, 2008).

Recently Qureshi et al. (2011) proposed a new cell extrinsic mechanism of inhibition by CTLA-4. By tracking GFP tagged CD86, it has been suggested that CTLA-4 binds to and internalises its ligands CD80 and CD86 from opposing cells through trans-endocytosis. The depletion of CD80 and CD86 then prevents T cell activation through CD28 (Qureshi et al., 2011).

TGF- β has a number of suppressive effects including suppressing the production of IFN- γ by Th1 cells, inhibiting IL-2 receptor expression and inducing apoptosis in T cells (Prud'homme and Piccirillo, 2000). TGF- β also exerts inhibitory effects on macrophages and B cells (Prud'homme and Piccirillo, 2000). The suppressive effect of TGF- β was demonstrated in a T cell transfer mouse model of colitis. Neutralising antibodies to TGF- β eliminated the protective effect of CD45RB^{low} T cells in CD45RB^{high} T cell mediated colitis suggesting that TGF- β plays a role in regulating T cell responses (Powrie et al., 1996). TGF- β exerts its effects by binding to the TGF- β receptor complex on the cell surface of target cells leading to the activation of SMAD proteins which transduce TGF- β signalling. Activated SMAD proteins translocate into the nucleus where they are targeted to specific genes by transcriptional factors (Massague, 2012, Heldin et al., 1997). SMAD proteins then recruit other enzymes that modify histones and remodel chromatin to regulate gene expression (Massague, 2012).

IL-10 is a cytokine secreted by Tregs under certain antigenic stimulation and has a suppressive effect by reducing T cell proliferation and the production cytokines such as IL-2 (Sundstedt et al., 2003, O'Garra et al., 2004). Through knocking out Treg specific IL-10 in mice it has been shown that IL-10 is important in controlling immune responses at exposed surfaces such as the lungs and colon but is not essential for controlling autoimmune disease (Rubtsov et al., 2008).

1.5.3 *Identification of Regulatory T cells*

Tregs contribute to 5-10% of the peripheral CD4⁺ T cell population in a healthy individuals (O'Garra and Vieira, 2003).

Flow cytometry is often used to isolate lymphocytes and the capacity to detect several markers at once on a cell surface improves the purity of a population. Early studies used high expression of CD25, the alpha chain of the IL-2R, to identify Tregs

(Sakaguchi et al., 1995, Stephens et al., 2001). Foxp3 has since been identified as the transcriptional regulator of Treg cells and often CD4+foxp3+ are identified as Tregs (Fontenot et al., 2003, Lee et al., 2007, Wang et al., 2011b). However foxp3 can be transiently expressed by activated T cells (Ziegler, 2007, Bernardo et al., 2012a, Wang et al., 2007).

Isolation of a pure Tregs population from human lymphoid tissue without fixing and risking damaging the cellular DNA by intranuclearly staining for foxp3, has proved to be challenging. Liu et al. (2006) discovered that low expression of CD127, the IL-7 receptor, correlated with high expression of foxp3 and CD127^{low}CD25^{high} CD4 T cells have a high suppressive function in peripheral blood. The inhibitory molecule, PD-1, has also been reported to be expressed on Tregs, especially after viral infection (Shen et al., 2011, Franceschini et al., 2009)

Miyara et al. (2009) identified three subsets of Tregs by their expression of CD45RA and foxp3. Resting Tregs express CD45RA and intermediate levels of foxp3, memory Tregs express intermediate levels of foxp3 and are CD45RA- and activated Tregs express high levels of foxp3 and are also CD45RA-. As the level of CD25 expression correlates with the level of foxp3 expression, CD25 expression can be used instead of foxp3 when identifying T reg subsets (Miyara et al., 2009).

1.6 B cells

B cells are part of the adaptive immune system and contribute to antigen specific immune responses. Maturation of B cells to plasma cells results in the secretion of antibodies that have a variety of functions including neutralisation of extracellular pathogens or toxins, complement activation and opsonisation. Alternatively, activated B cells can differentiate into memory B cells which can respond readily to re-exposure of antigen.

1.6.1 *B cell generation*

B cells arise in the bone marrow from common lymphoid progenitor cells (Kondo et al., 1997). During development the B cell receptor (BCR) is formed which gives the B cells its unique antigenic specificity. The BCR is made up of Ig heavy and light chains that are similar in structure to TCR chains (Bassing et al., 2002). The Ig heavy chains are made up of V,D and J segments whereas the Ig light chains are made up of only V and J segments (Bassing et al., 2002).

B cell development begins with the induction of the transcription factor E2A, which specifies a B cell fate and gives rise to progenitor B cells (Pro-B cell) (Singh et al., 2005). E2A regulates gene expression in the pro-B cell that results in Ig heavy chain gene rearrangement (Singh et al., 2005). Once a heavy chain has successfully rearranged it is tested by being expressed on the cell surface along with surrogate light chain to form a pre-B cell receptor (Nishimoto et al., 1991). If signalling occurs through the pre-B cell receptor then the B cells continues development as a precursor B cell (pre-B cell) and the Ig light chains are rearranged (Herzog et al., 2009). Once a successful light chain is rearranged it is paired with a μ chain and expressed on the cell surface along with the heavy chain as IgM (King and Monroe, 2000). At this stage the pre-B cell becomes an immature B cell and undergoes negative selection (King and Monroe, 2000). Only immature B cells with low affinity for self-antigens in the bone marrow continue with development (Nemazee, 2006). As the immature B cells are only tested against self-antigens in the bone marrow, the negative selection at this point results in central tolerance (Nemazee, 2006).

The immature B cells emerge from the bone marrow as new emigrant B cells and undergo negative selection resulting in peripheral tolerance (Samuels et al., 2005). It is suggested that tolerance to self-antigen results in the deletion of autoreactive B cells

or they enter an anergic state (Goodnow et al., 1988, Russell et al., 1991). However even after undergoing the peripheral checkpoint approximately 20% of mature naïve B cells expressed immunoglobulins that are HEp-2 reactive (Samuels et al., 2005).

The mature naïve B cells recirculate through lymphoid tissue where they may encounter antigen and mature to give rise to plasma and memory B cells. By using transgenic mouse models expressing hen egg lysozyme (HEL) and rearranged anti-HEL immunoglobulin genes it was demonstrated that self-reactive B cells that slip through the peripheral checkpoint usually have a short life span (Fulcher et al., 1996). However self-reactive B cells can mature and secrete antibody when sufficient T cell help is provided (Fulcher et al., 1996). This suggests that the regulation of autoreactive B cells in the periphery is to some extent regulated by T cell tolerance.

1.6.2 ***B cell activation***

The consequences of B cell activation depend on the nature of the immunising antigen. Antigens that include a protein component such as cholera toxin B, enable B cells to recruit cognate T cell help and are therefore called T cell dependent (TD) (Campbell and Munson, 1987, Cerutti et al., 2011). Antigens that do not have a protein component but which are able to activate B cells by their ability to bind an innate receptor, such as unmethylated CpG repeats in bacterial DNA, or by the repetitive nature of their antigenic structures, such as microbial polysaccharides, are referred to as T cell independent (TI) antigens (Cerutti et al., 2011, Peng, 2005). This thesis will focus on T cell dependent activation of B cells.

TD activation of B cells requires multiple signals. The first signal is from antigen binding to the BCR. The antigen is then internalised while bound to the BCR, degraded and presented as peptides in peptide:MHC class II complexes on the cell surface (Parker, 1993). Pre-primed cognate T helper cells that have TCRs specific for the

peptide presented by the B cell (Parker, 1993) deliver multiple signals including delivery of cytokines into the synapse and cross linking of CD40 by CD40 ligand (CD40L) expression (Jaiswal and Croft, 1997). CD40-CD40L signalling induces B cells proliferation and the upregulation of co-stimulatory molecules (Bishop and Hostager, 2003).

Activation of B cells causes B cells to proliferate along with T cells. Some of the B cells differentiate into plasmablasts and secrete antibodies encoded by germline immunoglobulin gene rearrangements. This forms a rapid response to pathogens but the plasma cells are short lived. Some activated B cells migrate to the follicle and either form or enter existing germinal centres (GC). Within the GC the B cells rapidly proliferate and undergo somatic hyper mutation (SHM) and class switching before differentiating into long lived plasma cells or memory B cells. This thesis will focus on the GC generated plasma cells (Shapiro-Shelef and Calame, 2005).

1.7 Formation of plasma cell precursors in germinal centre reactions

GCs are the sites of extensive B cell proliferation and immunoglobulin diversification to generate antibodies with potentially greater affinity for the antigen.

B cells upregulate CXCR5 when they are activated and some of these B cells retain the expression of CXCR5 (Zotos and Tarlinton, 2012). CXCR5 is the chemokine receptor for CXCL13. CXCL13 is produced by follicular dendritic cells (FDC) and expression of CXCR5 on the cell surface allows migration of a cell in the B cell follicle and GC (Gunn et al., 1998, Legler et al., 1998). The CXCR5⁺ activated B cells enter the B cell follicle along with antigen specific preT_{FH} cells, possibly originating from the interfollicular zone (Fuller et al., 1993, Kerfoot et al., 2011, Zotos and Tarlinton, 2012). After the initiation of a GC, a dark and light zone form (Zotos and Tarlinton, 2012). The

dark zone is proximal to the T cell zone and is where actively proliferating B cells reside, called centroblasts (Victora and Nussenzweig, 2012). In the dark zone the centroblasts undergo SHM and immunoglobulin class switching (Victora and Nussenzweig, 2012). The centroblasts then differentiate into centrocytes and are selected for high affinity for antigen (Victora and Nussenzweig, 2012).

The majority of centrocytes reside in the light zone and this is where selection of B cells with high affinity receptors generated by somatic hypermutation is mainly thought to occur. The light zone contains a network of FDC that retain antigen on their cell surface complex with complement and antibody. Many of the T_{FH} reside in the light zone (Victora and Nussenzweig, 2012, Zotos and Tarlinton, 2012). However, some proliferation of GC B cells can be observed within the light zone and less movement is seen between the light and dark zone than previously predicted. It has therefore been suggested that proliferation and selection may occur in both the light and dark zone (Zotos and Tarlinton, 2012).

Centrocytes sample antigen from the surface of FDCs by their altered BCR (MacLennan, 1994). The antigen bound to the BCR is internalised and presented to T_{FH} on the surface of the centrocyte as peptides:MHC class II complexes. This provides cell to cell contact and cytokines that contribute to the centrocyte survival (Vinuesa et al., 2009). If, after this process the centrocytes have high affinity for the antigen they may differentiate into plasma cells or memory cells and leave the lymphoid follicle (Zotos and Tarlinton, 2012). However if they do not produce high affinity antibodies they either die or may re-enter into the centroblast pool to start the process again (Fig 1.5 and 1.6) (Zotos and Tarlinton, 2012).

Germinal centre

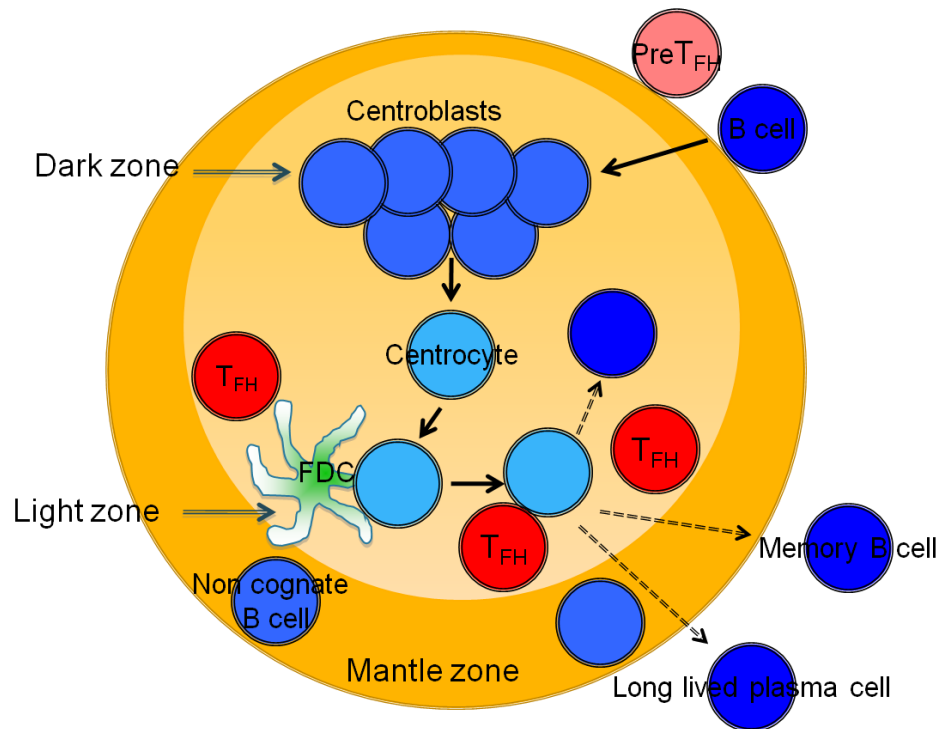


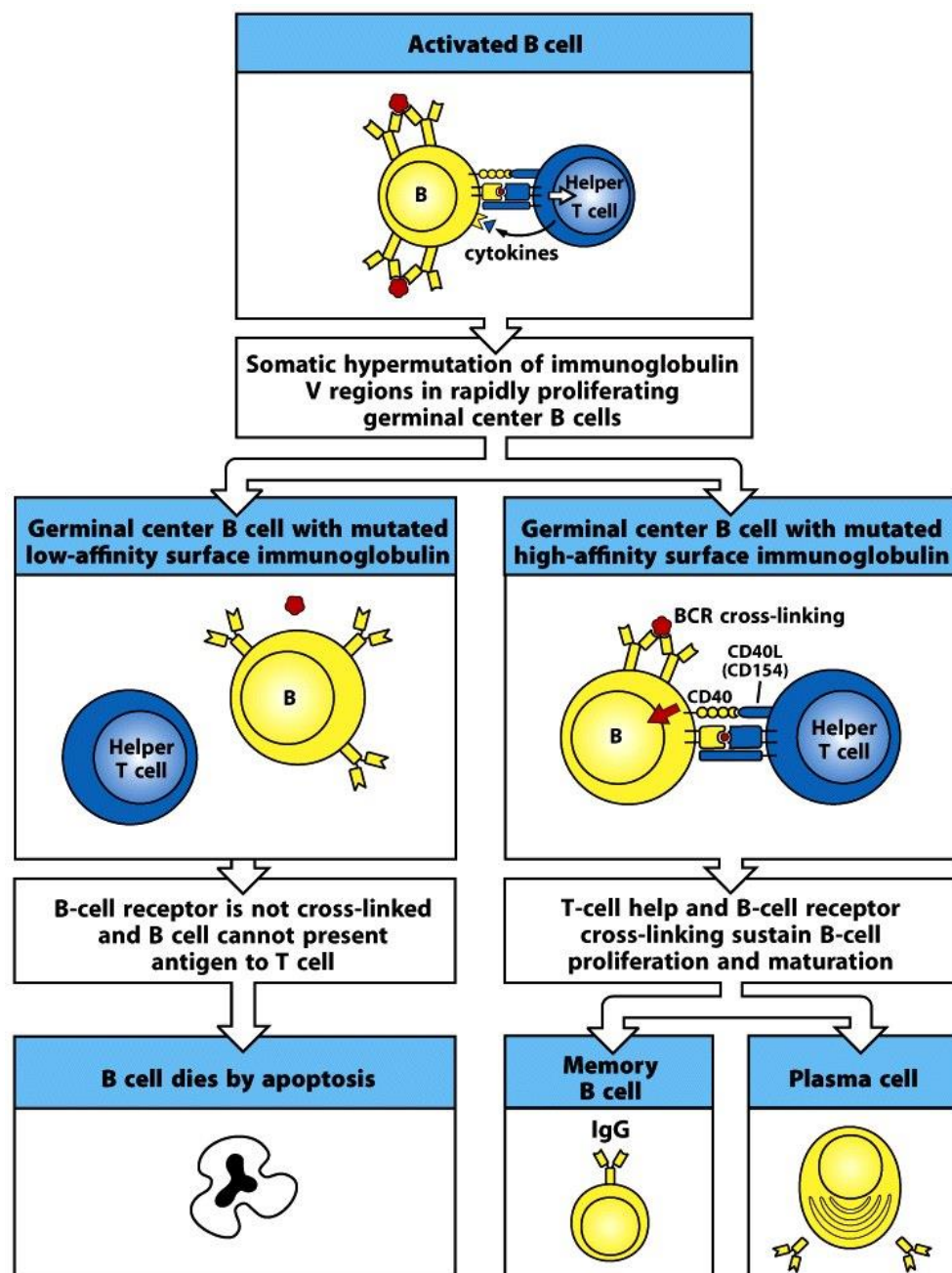
Figure 1-5 Cellular interactions in a Germinal Centre.

1.7.1 *Selection of germinal centre B cells*

The survival of high affinity B cells over low affinity B cells is thought to be due to a restriction of available antigen, cytokines and T cell interactions that creates a competitive environment. However a mathematical model and a mouse model that allows tracing of B cells expressing photoactivatable fluorescent proteins, suggests that competition for T cell interaction is key to the selection of high affinity B cells and not the availability of antigen (Meyer-Hermann et al., 2006, Victora et al., 2010).

T cells have been demonstrated to polarise to B cells with the highest density of peptide-MHC II complex expression (Depoil et al., 2005). Therefore centrocytes with high affinity BCRs are able to bind and process more antigen and therefore are more likely to receive help from T_{FH} (Zotos and Tarlinton, 2012). T_{FH} provide centrocytes

with a number of contact dependent signals. CD40L on the T cell binds to CD40 on the centrocyte. The outcome of CD40-CD40L signalling precisely within the germinal centre is not known but one possibility is that CD40-CD40L signalling causes the centrocyte to enter into the cell cycle (Vinuesa et al., 2010). GCs do not form in the absence of CD40:CD40L interaction and blocking CD40L signalling abolishes established GCs (Fig 1.6) (Han et al., 1995, Noelle et al., 1992).



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Figure 1-6 Germinal Centre B cells are selected for higher affinity by T_{FH} .

1.8 *T follicular helper cells*

T_{FH} are essential for the initiation of germinal centres and the formation of GC B cells (Vinuesa et al., 2010).

1.8.1 *T follicular helper cell development*

T_{FH} cells are thought to arise from naive CD4 T cells in response to receiving strong TCR signalling or repeated encounters with APC (Fazilleau et al., 2009, Baumjohann et al., 2011). This leads to the stable expression of the transcription factor Bcl-6 which in turn induces upregulation of CXCR5 (Baumjohann et al., 2011). The expression of CXCR5 allows migration of the T_{FH} cell into the GC (Yu et al., 2009a). Interaction with cognate B cells is also required to maintain T_{FH} in the GC (Qi et al., 2008). SAP deficiency prevents stable interaction occurring between T and B cells (Qi et al., 2008). In SAP deficient mice, T_{FH} still develop as they are still able to form interactions with APC but the T_{FH} can not be recruited or retained in the GC leading to a GC deficiency (Qi et al., 2008).

IL-21 is important in the development of T_{FH} cells as well as being highly expressed by T_{FH} cells and it has been suggested that IL-21 might act in an autocrine manner (Vogelzang et al., 2008). IL-21^{-/-} mice have defective T_{FH} generation due to insufficient expression of CXCR5 suggesting that IL-21 is need for high expression of CXCR5 on T_{FH} (Vogelzang et al., 2008).

It has recently been suggested that T_{FH} cells can also arise by T cell plasticity, for example from Treg in the gut-associated lymphoid tissue of mice (Lu et al., 2011, Tsuji et al., 2009). This will be discussed in more detail on chapter 4.1.

1.8.2 *Features of T follicular helper cells*

T_{FH} are defined by their residence in GCs and although several surface markers and the transcription factor Bcl-6 have been associated with T_{FH} , no defining marker has

been found. Several subsets of T_{FH} have already been identified including CD8 T_{FH} and follicular T reg cells (Tfr) (Linterman et al., 2011, Quigley et al., 2007). However, approximately 80% of GC T cells are the classical CD4 T_{FH} effector cells with B cell helper abilities and the main focus of this thesis will be on this subset of T_{FH} (Linterman et al., 2011). The surface markers associated with T_{FH} are CXCR5, PD-1, CD57, and ICOS though one marker alone is not sufficient to characterise T_{FH} .

1.8.2.1 CXCR5/CXCL13

CXCR5 expression allows migration towards the chemokine CXCL13. CXCL13 is produced by FDC which reside in the GC and B cell follicle and entry into this microenvironment is dependent on the expression of CXCR5 (Gunn et al., 1998). Without CXCR5, it has been shown in mice that the structure of lymphoid tissues are disrupted and GCs fail to form (Förster et al., 1996). Antigen activation through the TCR induces the upregulation of CXCR5 and migration to the T-B cell border. However not all CXCR5⁺ T cells enter the follicle and GC (Ansel et al., 1999). It is suggested that other interactions between the T cells and B cells enable a select few of CXCR5⁺ T cells to enter the follicle and then the GC where they can be defined as T_{FH} . T_{FH} can be distinguished from activated T cells at the T-B cell border, by their high expression of CXCR5 (Yu and Vinuesa, 2010). There are some discrepancies in the literature as to the phenotype of T_{FH} due to some groups defining T_{FH} as CXCR5⁺ and some as CXCR5^{high}. However it is clear that T_{FH} are only found in the CXCR5⁺ and not in the CXCR5⁻ compartment of T cells demonstrated by the ability of CXCR5⁺ T cells to induce antibody production. Therefore CXCR5 expression is a defining feature of T_{FH} (Schaerli et al., 2000).

The chemokine CXCL13 has also been reported to be expressed by T_{FH} . mRNA for CXCL13 is expressed by PD-1^{high} tonsil T cells and CXCL13 protein is detected within

these cells by IHC (Wang et al., 2011a, Yu et al., 2009b). It is possible that CXCL13 production by T_{FH} is induced once they have entered the GC and this may act in an autocrine manner to maintain their position or to recruit more cells.

1.8.2.2 CD57

CD57 was first found to be expressed by NK cells (Abo and Balch, 1981). However CD57 is expressed on a wide variety of cell types including neuronal cells, NK and NK T cells and T cells- both CD8 and CD4 (Focosi et al., 2010).

On NK cells, NK T cells, CD8 T cells and CD4 T cells, CD57 has been associated with replicative senescence (clonal exhaustion) where the cells are unable to proliferate but can still secrete cytokines (Brenchley et al., 2003). An increase in CD57 expression on lymphocytes is associated with chronic immune activation and a variety of diseases such as cancer, autoimmune diseases and HIV (Focosi et al., 2010).

Expression of CD57 on T_{FH} has been known for a long time but the function on T_{FH}, if any, is still unknown. Both CD57⁺ and CD57⁻ T_{FH} can be observed in GCs but in-depth analysis of the two population has not yet been done (Poppema et al., 1983). It has been suggested that CD57⁺ and CD57⁻ T_{FH} are functionally different with CD57⁻ fated to become Th2 memory cells (Johansson-Lindbom et al., 2003) or that the CD57⁻ T cells in GCs are precursors of the CD57⁺ T cells (Vinuesa et al., 2005). At first it was believed that only T cells positive for CD57 were able to stimulate B cells to produce antibodies (Kim et al., 2005). However subsequent studies have demonstrated that CD57⁺ and CD57⁻ T_{FH} have the same B cell stimulatory capacity but the precise function of the CD57⁻ T_{FH} is unknown (Rasheed et al., 2006).

CD57 expression by human T_{FH} in peripheral lymphoid tissues such as tonsil, spleen and lymph nodes has been described (Poppema et al., 1983, Rasheed et al., 2006), but there is little research into CD57 expression in GALT. CD57 is not expressed by

murine T_{FH} and therefore mouse model cannot be used to investigate CD57 (Zheng et al., 1996).

1.8.2.3 ICOS

Inducible co-stimulator (ICOS) was first described as a T cell specific cell surface receptor that enhanced T cell responses to antigens by increasing proliferation, cytokine secretion and antibody secretion (Hutloff et al., 1999). ICOS is a co-stimulatory molecule that modifies the T cell response to activation and its ligand, LICOS, is expressed on activated dendritic cells, monocytes and B cells (Murphy et al., 2008). ICOS is highly expressed on T_{FH} within GCs and T_{FH} are depleted in ICOS deficient patients and mice (Hutloff et al., 1999, Bossaller et al., 2006). However there are differences in the expression of ICOS between humans and mice. In humans T_{FH} have high expression of ICOS but in mice T_{FH} and non-T_{FH} express similar levels of ICOS (Yu and Vinuesa, 2010).

1.8.2.4 PD-1

PD-1 mRNA transcript was first isolated from murine cell lines undergoing programmed cell death and identified as a member of the immunoglobulin superfamily. It is the surface receptor for PD-L1 (B7-H1) and PD-L2 (B7-DC) and it is involved in the regulation of immune responses (Ishida et al., 1992, Latchman et al., 2001, Freeman et al., 2000). PD-1 expression can be induced in T cells and B cells by stimulation through the antigen receptor (Agata et al., 1996).

PD-1 is expressed in lymphoid tissues and particularly co-localises with T_{FH} (Iwai et al., 2002). This is thought to be a mechanism to control antibody production through preventing T_{FH} proliferation and their B cell helper activity (Wang et al., 2011a).

In mice, PD-1, PD-L1 and PD-L2 expression can be found on GC B cells and interaction between PD-1 on T_{FH} and PD-L on B cells are thought to contribute to high

affinity antibody production (Good-Jacobson et al., 2010). It is suggested that this pathway regulates the number of T_{FH} , therefore limiting the T_{FH} -B cells interactions to only B cells expressing high affinity BCRs so that a selective pressure is maintained (Hams et al., 2011). As it is suggested that PD-L-PD-1 pathway has a greater inhibitory effect on weaker TCR signalling, therefore T_{FH} cells with the higher affinity for the antigen will remain (Keir et al., 2008).

Without PD-L1/ PD-1 interaction, T_{FH} expand leading to an elevated Ig response. An increase in T_{FH} has been associated with many autoimmune diseases which demonstrates the importance of PD-1 in regulating the humoral response (Hams et al., 2011).

1.8.2.5 CD40L

CD40L is the ligand for the co-stimulatory molecule CD40. CD40L is part of the TNF superfamily and expression of CD40L is induced on activated T cells and is functionally important for their ability to activate B cells and macrophages (Murphy et al., 2008). CD40L is highly associated with T_{FH} cells and is key for their ability to promote the survival of antigen specific GC B cells as described earlier. This is demonstrated by hyper IgM syndrome in patients with deficiencies in the CD40L gene as there is a failure to produce class switched B cells (Allen et al., 1993). Also in mice, administration of anti-CD40L antibody prevented GC formation in response to TD antigen challenge (Han et al., 1995). CD40L has been shown to be rapidly upregulated on T_{FH} cells upon stimulation by transfer from the cytoplasm to the surface and therefore CD40L may not be expressed on resting T_{FH} (Casamayor-Palleja et al., 1995).

1.9 Lymphoid tissues

Secondary lymphoid tissues include lymph nodes, spleen and mucosa associated lymphoid tissues (MALT) and are the major sites for lymphocyte priming and the GC

reaction (Murphy et al., 2008). Different lymphoid tissues receive antigens from different tissue sites.

The spleen is not connected to the lymphatic system but instead the lymphocytes and antigens arrive via the blood (Cesta, 2006). The white pulp is the area of lymphoid tissue surround arterioles and where adaptive immune responses to blood borne pathogens takes place (Cesta, 2006, Mebius and Kraal, 2005).

In MALT, lymphocytes respond to antigens sampled from the mucosal surface. Antigens from the mucosa are sampled at specific sites along the epithelium where there are microfold 'M' cells (Neutra et al., 2001). The DC sample the antigens at the follicle associated epithelium and prime T cells in the subepithelial dome regions and the T cell zone of the follicle (Neutra et al., 2001). DC are able to open up the epithelial barrier but keep the epithelial integrity by the expression of tight junction proteins (Rescigno et al., 2001). In mice a subset of DC expressing CD103 migrate to the mesenteric lymph nodes after sampling antigen and induce tolerance instead of priming (Pabst et al., 2007). A population of DC similar to the CD103⁺ DC in mice have been identified in humans lymph nodes and may have the same function (Jaensson et al., 2008). Antigen presentation in the mesenteric lymph node is associated with tolerance induction by the generation of $\alpha 4\beta 7$ and CCR9 expressing iTreg, whereas antigen presentation by DCs in MALT is associated with priming (Pabst and Mowat, 2012). iTreg contribute to maintenance of immunological homeostasis in the gut and also oral tolerance.

MALT can be further sub characterised by the anatomical location of the lymphoid tissue. Gut associated lymphoid tissue (GALT) includes the appendix, Peyer's patches and isolated lymphoid follicles (ILF) along the colon and ileum. Peyer's patches are concentrated in the terminal ileum and are made up of clusters of follicles. ILF are single follicles distanced along the length of the intestine. In mice, ILF are derived from

cryptopatches in response to recognition of the microbiota by epithelial NOD (Bouskra et al., 2008). Nasal-associated lymphoid tissue (NALT) in humans consists of the Waldeyer's ring which is made up of the adenoids, and palatine tonsils (Brandtzaeg, 2011). Rodents have similar NALT structures to humans except they lack tonsils (Brandtzaeg, 2011).

Most of the plasma cell progeny generated by B cell activation generally occupies two different microenvironments; the intestinal lamina propria (LP) and the bone marrow. Plasma cells induced at peripheral lymphoid tissue (spleen, lymph nodes and tonsils) mostly localise in the bone marrow but would rarely enter the gut. The majority of plasma cells induced in GALT are imprinted with a set of homing receptors and chemokine receptors that permit them to return the intestinal immune effector site in the LP. Imprinting is dependent on the activity of local DCs (Stagg et al., 2002). GALT DCs express the enzyme retinaldehyde dehydrogenase (RALDH) that generates retinoic acid from vitamin A. Retinoic acid then upregulates $\alpha 4\beta 7$ integrin and chemokine receptor CCR9 on B cells and T cells activated in GALT that facilitate homing to the gut LP. Return to the LP effector site is mediated by binding of $\alpha 4\beta 7$ integrin to endothelial mucosal addressin cell adhesion molecule-1 (MAdCAM1) and in response to the epithelial derived chemokine CCL25 (Mora et al., 2003, Mora and von Andrian, 2009). Differential homing between the colon and small intestine is mediated in part by differential expression of CCR9 that mediates migration towards CCL25 that is produced mostly in the small bowel and CCR10 that mediates movement towards CCL28 that is produced mostly in the colon (Sundström et al., 2008, Kunkel and Butcher, 2003). In addition to cells derived from the gut that localise in the LP, some have also been shown to localise in the bone marrow (Mei et al., 2009). In contrast homing of plasma cells to bone marrow includes expression of CXCR4 that mediates homing in response to bone marrow derived CXCL12 (Kunkel and Butcher, 2003).

1.9.1 *Differences between NALT and GALT*

NALT is sometimes considered to be mid-way between mucosal and systemic immunity. Anatomically it is situated in a mucosal site on the oropharynx with a high antigenic load. However, it is covered by a stratified or pseudostratified epithelium and not the single epithelial layer that covers MALT throughout the gastrointestinal tract (Brandtzaeg, 2011). Tonsil high endothelial venules express peripheral lymph node addressin which is normally expressed in lymph nodes and binds to L-selectin on the naive lymphocytes and recruits the lymphocytes into the tissue (Brandtzaeg, 2011). In contrast, GALT high endothelial venules expresses MAdCAM-1 (Brandtzaeg, 2011). The tonsil lymphoid tissue generates plasmablasts that are around 55–72% IgG and 13–18% IgA (Brandtzaeg, 2011). This ratio of plasmablasts is similar in other peripheral lymphoid tissues and is distinct from GALT which generates a high proportion of IgA plasmablasts. Therefore tonsil, due to having features of a systemic lymphoid tissue, is considered to be part of the peripheral immune system in this thesis.

1.10 *Antibody generation in the gut.*

1.10.1 *Antibody distribution*

In humans, the gut mucosa contains approximately 80% of all antibody secreting cells (Brandtzaeg et al., 1999). The features of the antibodies secreted at the gut mucosa differ substantially from those circulating in the blood and extra cellular fluid. IgG is the main class of antibody circulating in the blood, however in the gut and other mucosal surfaces, a high proportion of IgA is secreted. The secreted IgA in the gut is mainly dimeric. IgA is able to dimerise due to a cysteine residue at the tail end of the C region. Disulphide bonds from the cysteine residues form between the two IgA monomers and a J chain. Dimerisation of IgA is required for its secretion (Murphy et al., 2008).

The ratio of IgA1 to IgA2 differs in the different anatomical sites. In peripheral lymphoid tissues such as the spleen and lymph nodes, the majority of IgA produced is IgA1. However in the gut the proportion of IgA2 increases and so that IgA1 only constitutes approximately 60% of the IgA secreted in the ileum and approximately 36% in the colon (Kett et al., 1986, Brandtzaeg et al., 1999).

One of the hypotheses to explain the difference in the distribution of IgA subclass through the gut is the difference in the distribution of antigens. Antibodies against food antigens tend to be IgA1 and therefore are found at a higher frequency in the upper GI tract whereas IgA2 tend to be against gram negative bacteria which is more abundant than food antigens in the colon (Brandtzaeg et al., 1999). It has been suggested that class switch to IgA2 in the colon is driven by a proliferation inducing ligand (APRIL) produced by colonic epithelium in response to the TLR ligation by bacterial flagellin from the lumen of the colon (He et al., 2007). This will be discussed in more detail below.

1.10.2 *AID in the generation of IgA plasma cells*

Activation-induced cytidine deaminase (AID) is important for antibody diversification and enables somatic hypermutation of germline immunoglobulin sequences and class switch recombination. AID deaminates cytidine to uridine. The uridine can be replicated over if the cell divides, in which case it will appear as a C to T (or G to A) transition. Alternatively it may be excised by the base excision repair enzyme uracil DNA glycosylase, leaving an abasic site where mutations can be introduced. Further enzymatic activity may generate a double strand break allowing class switch recombination (Murphy et al., 2008). Mouse models of k/o and mutated *aicda* show the importance of AID in the generation of functional IgA. *Aicda* k/o mice have a deficiency in both CSR and SHM and therefore no IgA⁺ B cells were generated

in AID K/O mice. As a result of no IgA being secreted into the intestinal lumen, the gut bacteria become dysregulated with an accumulation of anaerobes. This in turn caused a continuous stimulation of B cells which resulted in large protruding lymphoid follicles in the gut (Fagarasan et al., 2002). Mice carrying a mutated form of AID which is able to initiate CSR but not SHM showed a similar phenotype to *aicda* k/o mice with enlarged lymphoid follicles in the gut. Therefore this shows that not only is the secretion of IgA important in gut homeostasis but mutations introduced by SHM in GALT is also needed for the regulation of gut flora (Wei et al., 2011).

1.10.3 ***Lamina propria as an effector site and not an inductive site***

LP B cells are mainly of a differentiated memory phenotype, or immediate precursors of plasma cells demonstrated by the expression of CD27 on their cells surface in humans which contributes to the idea of the LP as an effector site. The CD27⁺ B cells in the LP tend to be CD20⁻, IgA⁺ whereas in the GALT the majority of B cells are CD20⁺, IgM⁺ (Farstad et al., 2000).

The CD27⁺ SIgA⁺ memory B cells were demonstrated to be able to expand and proliferate in response to selected stimuli *in vitro* and therefore mount a response to a pathogen at the effector site (Farstad et al., 2000). Evidence of proliferation in LP has been suggested by PCR based studies but not cell and tissue based studies (Boursier et al., 2005, Yuvaraj et al., 2009, Di Niro et al., 2012).

Fagarasan et al. (2001) suggested that in mice plasma cells are able to undergo CSR in the LP. They showed that a proportion of LP B220⁺IgA⁺ plasma cells had recently classed switched by the presence of AID and circular DNA transcripts which are looped out during CSR (Fagarasan et al., 2001). It is possible that these cells are the mice equivalent to the CD28⁺ CD19⁺IgA⁺ plasma blasts found in humans (Farstad et al., 2000). B220⁺IgM⁺ cells from AID^{-/-} mice were shown to be able to undergo CSR to

IgA when AID was transfected into the cells and this process was dependent on LP stromal cells, suggesting that the LP is an important inductive site (Fagarasan et al., 2001). This has since been shown to most probably be due to contamination with B cells from ILF when sampling LP. When the LP was reinvestigated in mice after careful removal of all organised lymphoid tissue, no evidence of AID transcripts was found (Shikina et al., 2004).

1.10.4 *Induction of an immune response in the gut*

The intestinal IgA response is produced through a germinal centre response in humans where AID is expressed and CSR can occur. Plasma cell precursors are imprinted as described previously and migrate to the effector site of the LP. The involvement of T cells (T_{FH} cells) in the germinal centre response is well characterised. However it has been demonstrated there are IgA plasma cells residing in the LP of the gut in CD40 deficient mice. Therefore IgA production can be induced independent of cognate T cell interaction. This T cell independent pathway of generation of IgA plasma cells was demonstrated to occur through ILF but not PP and is dependent on ROR γ t LTi cells which are needed for ILF formation in mice. Therefore the T cell independent induction of the IgA response in the gut still requires secondary lymphoid tissue (Tsuji et al., 2008).

In CD40^{-/-} mice CSR to IgA is still able to occur but is restricted to GALT in the absence of GCs and is suggested to be T cell independent as the CD40L-CD40 interaction in lymphoid tissues is normally from CD40L on T cells (Korthauer et al., 1993). Interestingly, though CSR is able to occur in these mice, the SHM rate is very low and this is reflected in the inability to raise an affinity matured response to T cell dependent pathogens (Bergqvist et al., 2010, Bergqvist et al., 2006). There is evidence that suggests that induction of IgA plasma cells in the human gut can also be T cell

independent. In humans it is thought that T cell independent class switching can be initiated by DCs and is dependent on BAFF and APRIL but independent of CD40L as it is in mice (Litinskiy et al., 2002, He et al., 2007).

It has been suggested that the LP B cells are not solely derived from GALT in mice, but that there is also a substantial contribution from the peritoneal B cell population (Bos et al., 1996). However, in the human gut LP the presence of peritoneal derived B1 cells is uncertain. Some groups have claimed to have found populations of B cells mirroring the peritoneal derived B1 population in mice but their contribution to the LP B cell population is a lot smaller than in mice (Farstad et al., 2000). Others have seen no evidence for this in humans (Boursier et al., 2002).

1.10.5 *Specificity of gut antibodies*

Engagement of the BCR on B cells has been shown to be crucial for the GC response in the gut (Barone et al., 2011). TLR mediated signals may be involved, but no more than in peripheral immune responses (Barone et al., 2011). It has been suggested that in GALT the specificity of the BCR for antigen is not such a stringent requirement for GC formation as it is in the periphery. Therefore this would generate mature B cells which are able to respond to a range of antigens very quickly and which would reside in the effector sites of the gut. This would result in a diverse population of B cells and not necessarily a specific response to antigen (Casola et al., 2004).

Monoclonal antibodies made from IgA secreting cells originating from PP of unimmunised, pathogen free mice, were able to bind to a variety of antigens. These polyreactive ‘natural’ antibodies which are continually produced to protect the host from commensal bacterial like natural IgM in the serum (Wijburg et al., 2006, Shimoda et al., 1999). But interestingly this polyreactivity includes autoreactive antibodies in the gut of healthy humans. However, the ability to generate highly specific IgA through a

gut mucosa response was demonstrated by Di Niro et al. (2010) who isolated IgA secreting plasma cells specific for rotaviruses from the gut and tested antibody specificity from single cells. In the human gut 25% of the antibodies produced are innate polyreactive antibodies from plasma cells and the rest are specific (Benckert et al., 2011, Di Niro et al., 2010).

However, regardless of the specificity of the antibodies, the majority of intestinal plasma cells have been through organised lymphoid tissue such as PP and ILF as the variable regions of the immunoglobulin genes encoding the secreted immunoglobulin was heavily mutated by somatic hypermutation (Barone et al., 2011, Benckert et al., 2011). As some of the polyreactive antibodies are also somatically mutated this leads to the conclusion that autoreactive antibodies are a product of the GC response.

The balance between specificity and autoimmunity in germinal centre responses has been shown to be regulated by T_{FH} (Linterman et al., 2009). It has recently been shown, since the work for this thesis was initiated, that T_{FH} have a crucial role in the determining the specificity of IgA responses in mice (Kawamoto et al., 2012).

1.11 Inflammatory Bowel Disease

The term ‘inflammatory bowel disease’ (IBD) refers to a group of chronic inflammatory conditions that mainly affect the gastrointestinal tract. The main types of IBD are ulcerative colitis (UC) and Crohn’s Disease (CD) (Xavier and Podolsky, 2007).

UC affects only the colon and the inflammation is diffuse and extends proximally from the rectum. The colonic mucosa becomes infiltrated by neutrophils and lymphocytes. Superficial crypt abscesses form and the mucosa thickens (Xavier and Podolsky, 2007).

CD can affect any part of the gastrointestinal tract but the inflammation extends through the full thickness of the bowel wall and is segmental. CD can also involve the ileum as well as the colon. Aggregations of macrophages form granulomas as a component of the inflammatory lesion (Xavier and Podolsky, 2007).

1.11.1 ***Factors contributing to the pathogenesis of IBD***

A complex interplay of genetics, environmental factors and the host immune system contribute to the development of both CD and UC. Recent analysis of several genome wide association studies (GWAS) found over 25,000 SNPs and 110 genetic risk loci associated with CD, UC or IBD in general (Jostins et al., 2012). 50 loci are shared between CD and UC suggesting that they have similar disease pathways (Jostins et al., 2012). SNPs in genes involved in innate immunity and autophagy such as *ATG16L1*, *IRGM* and *NOD2* are associated CD (Kaser and Blumberg, 2011). The profile of risk alleles in UC includes the *IL-2/IL-21* loci and several SNPs in IBD are also associated with autoimmune diseases such as Type I diabetes (Festen et al., 2009, Todd et al., 2007). Genetic loci associated with IBD also overlap with ankylosing spondylitis and psoriasis and the loci associated with IBD are enriched for genes associated with primary immunodeficiency, in particular Mendelian susceptibility to mycobacterial disease (MSMD) (Jostins et al., 2012). These risk loci highlight the role of microbes and host responses in the development of IBD.

Several other studies have also implicated intestinal microbiota in the development of IBD. The human gut contains over 10^{14} micro-organisms and they are important for the development of both the systemic and intestinal immune response (Kaser et al., 2010). Microbiota is able to both induce an immune response and also prevent an inflammation (Round and Mazmanian, 2009). There is increasing evidence that microbiota shape the immune system just as the immune system determines the

composition of the colonising bacteria. For example germ free mice have small peyer's patches and fewer IgA+ lamina propria plasma cells suggesting that the microbiota is needed in part for the development of GALT (Macpherson et al. 2001). In a gnotobiotic mouse model it was demonstrated that IgA specific to the microbiota prevented an innate inflammatory immune response to the bacteria so that the microbiota could continue to colonise the host (Peterson et al., 2007). Due to the synergistic relationship between microbiota and the immune system, dysbiosis of the microbiota could in part contribute to the development of IBD (Round and Mazmanian, 2009). The role of microbiota in IBD is demonstrated by the dependence of microbiota in mouse models of IBD and differences in the composition of microbiota between IBD patients and healthy individuals have been observed (Damman et al., 2012). Faecal microbiota transplantation has been used to treat several patients with IBD in an attempt to recolonize the intestinal tract with microbiota from a healthy individual. The majority of patients who received a faecal microbiota transplant remained in remission for several months and in some cases years (Damman et al., 2012).

Other environmental factors such as smoking can affect the probability of developing disease in UC and CD differently. Whereas smoking is protective in CD, appendectomy is thought to be protective in UC and will be discussed in more detail below (Danese et al., 2004). This suggests that events in the appendix might be directly involved in the pathogenic process in UC, or alternatively the benefit may be purely a consequence of removing a mass of active lymphoid tissue.

The host immune defences also play a crucial role in IBD. Both the innate and adaptive immune responses have been shown to be altered in IBD. Paneth cells are found within the epithelium of the small intestine and secrete antimicrobial peptides such as α -defensins and are part of the innate intestinal host defences (Kosłowski et al.,

2010). 30-40% of CD patients have polymorphisms in *NOD2* and some *NOD2* polymorphisms are thought to effect paneth cell differentiation and the secretion of antimicrobial defensins (Kaser et al., 2010, Koslowski et al., 2010). There is a large body of research into the role of the adaptive immune system in IBD. The changes observed in T cells and B cells are outlined below.

1.11.2 ***T cells and cytokines in inflammatory bowel disease.***

The chronic inflammation in IBD is associated with the production of cytokines produced by different T cell subsets.

CD has been described as a Th1 mediated disease due to the prevalence of Th1 associated cytokines such as IFN- γ and TNF α secreted by LP T cells. Whereas in UC cytokines secreted by LP T cells include IL13 leading to UC being considered a Th2 mediated disease (Fuss et al., 1996, Sarra et al., 2010b).

An increase in intestinal IL-12 is observed in CD and IL-12 was first thought to drive the aberrant Th1 response associated with CD (Matsuoka et al., 2004). The source of IL-12 in CD is in part intestinal DCs (Hart et al., 2005). However, recently Th17 cells and IL-23, have been implicated in the pathogenesis of CD and UC (Strober and Fuss, 2011). In a mouse models of *helicobacter hepaticus* induced T cell dependent colitis, it was demonstrated that more severe intestinal inflammation was dependent on IL-23 more than IL-12 (Kullberg et al., 2006). IL-23 deficient mice demonstrate the role of IL-23 in the development of Th17 cells and IL-23 also promotes T cell proliferation and IFN- γ production (McGeachy et al., 2009, Oppmann et al., 2000). Kobayashi et al. (2008) demonstrated that IL-23p19 mRNA expression was upregulated in both CD and UC and observed a higher increase in IL-17 mRNA in UC LP T cells and a higher increase in IFN- γ mRNA in CD LP T cells and also both UC and CD mucosal T cells produced the same high level of IL-17 when stimulated with anti-CD3 and anti-CD28

compared to normal controls. However, Sakuraba et al. (2009) observed a higher production of IL-17 by T cells from MLNs in CD compared to UC. Anti-IL-12p40 antibodies have been shown to improve symptoms in CD patients (Mannon et al., 2004). However, as both IL-12 and IL-23 share the p40 subunit, the beneficial effects of this antibody could be from neutralising either or both of these cytokines (Oppmann et al., 2000).

Mouse models of IBD and studies of human IBD have suggested that numerical or functional deficiencies in Tregs could contribute to the pathogenesis of IBD. In SAMP1/YitFc (SAMP) mice that spontaneously develops ileitis similar to human CD, Tregs have been demonstrated to be functionally deficient *in vivo* (Ishikawa et al., 2012). Mice lacking Treg derived IL-10 develop spontaneous colitis, while transfer of Tregs into the CD4⁺ CD45RB^{high} T cell mouse model of colitis eliminated disease (Mottet et al., 2003, Kuhn et al., 1993). However human studies on Tregs in IBD are inconsistent and will be discussed in more detail in chapter 5.1.

1.11.3 ***Dysregulation of B cells in inflammatory bowel disease.***

Dysregulation of the mucosal B cell response can be seen in IBD. SIgA normally elicits immune exclusion of pathogens to maintain the integrity of the gut epithelial barrier. However in IBD lesions, both IgA⁺ and IgG⁺ plasma cells are increased but with a particular disproportionate increase in the production of IgG (Brandtzaeg, 2010).

In UC the increase in IgG is predominantly due to an increase in IgG1 whereas in CD, there is an overall increase in all IgG subclasses and in particular IgG2 (Scott et al., 1986).

Serum antibodies to particular microbial and auto antigens are associated with IBD and can be used as a diagnostic tool. In UC several autoantibodies have been identified,

including perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) in patients serum and antibodies to tropomyosin 5 in colonic epithelial cells (Hibi et al., 1990, Targan et al., 1995, Onuma et al., 2000, Abad et al., 1997).

Systemic anti-*Saccharomyces cerevisiae* antibodies (ASCA) are a feature of CD but not UC. ASCA are thought to be against components of the *S. cerevisiae* cell wall mannan (Sendid et al., 1996). Landers et al. (2002) reported that 55% of CD patients had serum antibodies to bacterial outer-membrane porin C (OmpC) found on *E. coli* and anti-cBir1 antibodies reactive to *Clostridium* flagellin are associated with a subset of CD patients with more complicated disease (Targan et al., 2005).

Recently genetic variants in autophagy and microbial pattern recognition genes have been linked to the production of anti-microbial antibodies in CD. In particular variants in *NOD2* and *ATG16L1* are associated with ASCA production implicating autophagy pathways in the development of these antibodies (Murdoch et al., 2012). The presence of these antibodies in the serum of patients with IBD reflects the breakdown of immunological tolerance but their relevance to the pathogenesis of the disease has not yet been established.

It has been suggested that the inflammatory response in IBD is associated with a disturbance in lymphocyte traffic and the alteration in profile of antibodies secreted described above is consistent with this. Lymphocyte entry into tissue compartments depends on interactions between the lymphocytes and venular endothelium involving, integrins, selectins and chemokines and their receptors (Hart et al., 2010). The homing properties of lymphocytes are orchestrated by DCs and differences in the imprinting of lymphocytes by DCs has been described in IBD (Bernardo et al., 2012b, Stagg et al., 2002).

In IBD an influx of lymphocytes is observed in the effected intestinal mucosa and $\alpha 4\beta 7$ is increased on circulating activated T cells in CD patients (Meenan et al., 1997). Antibodies against $\alpha 4$ have been tested in the treatment of CD and therefore has implicated aberrant gut homing as possibly contributing to the disease (Ghosh, 2003).

1.12 The Appendix

The human appendix is a tubular organ of approximately 8-10 cm, attached to the caecum and is commonly thought to be a vestigial organ (Humes and Simpson, 2006). It contains a large amount of lymphoid tissue which is part of the GALT. There has been very little research into the immunological role of the human appendix, however a higher number of IgG+ B cells have been observed in the appendix compared to the colon suggesting that perhaps the two sites are responding to different antigens or are generating different immune responses (Bjerke et al., 1986). In some mammals such as rabbits, the appendix is proposed to be a primary site of B cell generation and plays a key role in mucosal responses. This is demonstrated by a reduction of intestinal antibodies after neonatal appendectomy (Dasso and Howell, 1997). However, this does not seem to be the case for the human appendix as no detrimental changes to the mucosal immune system has been reported after removal of the appendix as treatment for appendicitis.

1.12.1 Appendicitis

Acute appendicitis is the inflammation of the appendix and accounts for 40,000 hospital admissions each year in England. It is most common in individuals between 10 and 20 years of age (Humes and Simpson, 2006). The cause of appendicitis is still unknown but it is thought to be a combination of factors including diet and obstruction (Humes and Simpson, 2006).

Acute appendicitis is also thought to have a genetic element as a significant number of patients with acute appendicitis had a family history of appendectomy compared to surgical controls (Basta et al., 1990). In this cohort the relative risk of developing appendicitis was 10 and the heritability was calculated to be 56% (Basta et al., 1990).

Acute appendicitis is most commonly treated by early appendectomy however studies are being conducted into treating uncomplicated acute appendicitis with antibiotic therapy (Paajanen et al., 2013).

1.12.2 *The role of the appendix in the development of UC*

A negative correlation between appendectomy and the development of UC has been demonstrated in several studies. Patients who underwent an appendectomy for appendicitis before the age of 20 had a lower risk of developing UC but patients who had an appendectomy of non-specific abdominal pain were as likely to develop UC as controls (Andersson et al., 2001). Also appendectomy prior to the onset of disease is rare in patients with UC (Andersson et al., 2001). One explanation for the negative correlation between appendectomy and the development of UC is that removing the appendix which contains a large amount of GALT, modulates the mucosal immune system (Radford-Smith et al., 2002).

Several studies have demonstrated immunological associations between the appendix and inflamed tissue in UC. It has been observed that a number of UC patients with distal colitis also had peri-appendiceal red patch (PARP). The presence of PARP was also associated with progression to more extensive disease (Rubin and Rothe, 2010, Matsumoto et al., 2002). T cell clones have been identified in both the appendix and the inflamed colon of patients with UC and CD. Also autoantibodies associated with UC were found in the appendix of TNF α mutant mice leading to the possibility that the

autoantibodies could be generated in the appendix (Chott et al., 1996, Radford-Smith et al., 2002).

In light of these studies, several patients with UC have undergone appendectomies as treatment for the disease. The beneficial effects of this surgery seem to be variable. Okazaki et al. (2000) reported that a patient with UC with inflamed appendix and rectum, improved significantly after an appendectomy and remained in remission 3 years post-surgery. Bolin et al. (2009) reported that 27 out of 30 patients with ulcerative proctitis who underwent an appendectomy had an improved clinical activity index after surgery. However for 3 of the patients appendectomy did not improve their symptoms (Bolin et al., 2009).

Overall there is mounting evidence to suggest that the appendix plays a vital role in the development of UC and possibly could even be the origin of the dysregulated immune response.

1.13 Aims

The experiments in this thesis aim to investigate the properties of T_{FH} in the gut compared to the peripheral immune system, the possibility of a developmental relationship between T_{FH} and Treg, and the profile of these immunomodulatory T cells in inflammatory bowel disease. Ulcerative colitis includes an autoimmune component and this was considered to be of particular interest. The specific aims of the three chapters of results are as follows:

In chapter 3 the frequency and functional parameters of T_{FH} will be explored and compared between peripheral lymphoid tissues and GALT.

In chapter 4 the possibility of a developmental relationship between T_{FH} and Tregs will be explored by sequence analysis of rearranged TCR genes.

In chapter 5 the frequency and distribution of T_{FH} and Treg in tissue and peripheral blood from IBD patients will be analysed.

2 Chapter 2 Methods

2.1 Samples

All fresh peripheral blood and tissue was taken from healthy individuals and cancer and IBD patients with informed consent. Consent was obtained under the ethically approved projects 10/H1111/014 and 04/Q0702/81.

Tonsil specimens were collected from Guy's Hospital ENT day surgery unit from patients undergoing a tonsillectomy but who were otherwise healthy at the time of surgery. Tonsils were removed from patients with sleep apnoea or recurrent tonsillitis but all tonsils used were not inflamed at the time of surgery. Paired blood was also collected from some of the patients before surgery.

Healthy control blood was obtained from both male and female individuals who were well at the time of donation.

Patients who attended the IBD clinic at Guy's Hospital were invited to take part in the study by donating blood after informed consent. All patients had either CD or UC (see table 2.1 and 2.2). Disease activity was assessed clinically at the time of donation in each case. Active CD was indicated by a Harvey Bradshaw index (HBI) of ≥ 5 and active UC was defined with a Simple Colitis Clinical Activity index (SCCAI) of ≥ 5 (Harvey and Bradshaw, 1980, Walmsley et al., 1998). Disease activity was also corroborated by endoscopic (colonoscopy) or radiological (small bowel MRI) assessment close to the time of blood sampling. Patients with inactive disease had no symptoms at the time of sampling.

Fresh cancer appendix was obtained from patients with bowel cancer undergoing a right hemicolectomy to remove the cancerous tissue but where the appendix itself and surrounding tissue were not cancerous. Fresh IBD appendix were obtained from patients

with a clinical diagnosis of either CD or UC and were undergoing a right hemicolectomy. Patients gave informed consent before surgery. A small section of the appendix not needed for diagnosis was removed from the surgically excised specimen by pathologists.

Paraffin-embedded tissues were retrieved from the archives of the Histopathology Department of the Guy's and St Thomas' Hospital Trust or were from previous research projects stored under REC approval 04/Q0702/81. In chapter 5 paraffin embedded tissue from IBD and cancer patients who had undergone a right hemicolectomy was analysed. A diagnosis of CD or UC was made based on histological analysis of the resected tissue. Blocks of ileum and colon of involved but not acutely inflamed tissue were selected from the tissue available from IBD patients. For the cancer patients, tissue blocks furthest away from the tumour were selected. As the resected specimens varied in length in all patient groups, the specific section of ileum and colon selected for analysis varied between patients. The whole appendix was contained within a single block for all patients and whole tissue sections were analysed by IHC.

Sample name	Date of birth	Sex	CD - location	CD - behaviour	Active /Remission	Smoking status	Current medication
CD301	09/04/1985	m	L3	B1	Remission	non-smoker	antibiotics (tapering budesonide)
CD302	11/11/1991	m	L2	B1	Remission	non-smoker	azathioprine, allopurinol, adalimumab
CD303	30/04/1988	f	L2	B1	Remission	non-smoker	nil
CD304	15/05/1985	f	L1	B1	Active	current	prednisolone
CD305	16/11/1978	f	L2	B1	Remission	non-smoker	nil
CD306	28/10/1967	m	L1	B2	Remission	ex-smoker	tapering budesonide
CD307	06/03/1984	f	L1	B2	Remission	non-smoker	nil
CD308	20/07/1983	f	L3	B1	Remission	current	azathioprine
CD309	19/08/1978	f	L1	B1	Remission	ex-smoker	nil
CD310	21/08/1965	f	L3	B2	Remission	non-smoker	methotrexate
CD311	21/03/1971	m	L3	B3	Remission	non-smoker	azathioprine, infliximab
CD312	21/04/1980	m	L3	B1(p)	Remission	ex-smoker	adalimumab
CD313	02/12/1988	m	(perianal)	(p)	Remission	non-smoker	nil
CD314	02/06/1968	f	L2	B1(p)	Remission	non-smoker	methotrexate, infliximab
CD315	30/11/1967	f	L2	B1(p)	Active	current	nil
CD316	09/09/1966	m	L3	B2	Active	non-smoker	nil
CD317	18/08/1980	f	L2	B1(p)	Remission	ex-smoker	nil
CD318	10/07/1989	m	L1	B1	Active	non-smoker	adalimumab
CD319	15/06/1982	m	L2	B1	Active	non-smoker	prednisolone
CD320	01/09/1986	f	L3	B2	Active	current	azathioprine
CD321	11/10/1982	f	L2	B1	Active	ex-smoker	prednisolone
CD322	28/09/1987	m	L2	B1	Active	non-smoker	prednisolone, adalimumab, mercaptopurine
CD323	07/02/1990	f	L3	B1	Active	ex-smoker	prednisolone
CD324	29/04/1985	m	L1	B2	Active	non-smoker	azathioprine, budesonide
CD325	31/08/1990	f	L1	B2	Active	non-smoker	prednisolone
CD326	26/01/1963	f	L2	B1(p)	Active	current	prednisolone

Table 2-1 Patients with CD that donated blood samples analysed in Chapter 5

Sample name	Date of birth	Sex	UC - extent	Active/ Remission	Smoking status	Current medication
UC301	12/07/1968	m	E2	Active	non-smoker	prednisolone, azathioprine, mesalazine
UC302	28/08/1964	m	E2	Remission	ex-smoker	azathioprine
UC303	22/09/1970	m	E1	Remission	current	mesalazine
UC304	17/04/1968	m	E3	Remission	non-smoker	mesalazine
UC305	19/03/1988	m	E2	Remission	non-smoker	nil
UC306	31/12/1940	m	E2	Remission	current	mesalazine
UC307	21/03/1984	m	E2	Active	ex-smoker	nil
UC308	08/10/1944	m	E3	Remission	ex-smoker	nil
UC309	25/10/1984	f	E1	Remission	smoker	mesalazine
UC310	06/11/1968	m	E2	Active	non-smoker	mesalazine
UC311	17/11/1982	m	E3	Remission	non-smoker	azathioprine
UC312	29/11/1987	f	E2	Remission	non-smoker	nil
UC313	17/11/1987	f	E2	Active	current	azathioprine, ciclosporin
UC314	13/12/1981	m	E3	Remission	current	nil
UC315	11/02/1939	f	E2	Remission	non-smoker	nil
UC316	07/07/1981	m	E2	Remission	non-smoker	mesalazine
UC317	23/07/1983	f	E2	Active	non-smoker	mesalazine
UC318	10/04/1989	f	E2	Active	non-smoker	mesalazine
UC319	20/07/1958	m	E3	Remission	non-smoker	mesalazine
UC320	27/06/1977	f	E2	Active	ex-smoker	prednisolone
UC321	09/07/1970	m	E2	Active	ex-smoker	mesalazine
UC322	24/11/1973	f	E3	Active	non-smoker	prednisolone
UC323	02/05/1979	m	E1	Active	ex-smoker	mesalazine
UC324	27/04/1981	f	E2	Active	non-smoker	infliximab
UC325	20/12/1967	m	E2	Active	non-smoker	nil

Table 2-2 Patients with UC that donated blood samples analysed in Chapter 5

2.2 Solutions

2.2.1 Solutions in Immunohistochemistry

2.2.1.1 TBS wash

6.05g Trisma base (sigma), 80g sodium chloride and 38ml 1M HCl was dissolved in 10l dH₂O and mixed well. The solution was adjusted to pH 7.6.

2.2.2 Solutions in Flow cytometry

2.2.2.1 2% FACS Buffer

2ml Heat inactivated 0.2µm filtered FCS and 1ml Tris EDTA (Fluka) were added to 47ml sterile 1xPBS and kept at 4°C until use.

2.2.2.2 Medium

500ml RPMI Medium with GlutaMax (gbco) was fortified with 10% FCS and 1% penicillin-streptomycin.

2.2.3 Solutions in Electrophoresis

2.2.3.1 2.6.3 10x TBE buffer

108g of trizma base (sigma), 55g boric acid and 8.3g EDTA (sigma) were dissolved in 1l dH₂O by mixing vigorously.

2.2.3.2 Agarose gel electrophoresis

PCR products were run on a 3% or 1.5% agarose gel. Agarose gel was prepared with 6g or 3g 3:1 agarose (Flowgen) and 200ml 1x TBE buffer. The gel was heated in a microwave on a high setting for 4 min or until all bubbles had dispersed. 1µl 50µg/ml ethidium bromide was added to the liquid gel before pouring. The gel was left to set for 30 min. 6µl of PCR product was mixed with 2µl of dye, and then loaded into gel. 6µl of

phiX174 DNA markers (Promega) was used to identify the size of products. The gel was run at 125V for 60 minutes in 1x TBE before being visualised under UV light.

2.2.3.3 10% Polyacrylamide mini-gel

10% Polyacrylamide mini-gel was used to visualise for low molecular weight DNA samples. 13ml of dH₂O, 2ml of 10x TBE, 5ml of Acrylamide 37:1 ratio (Sigma), 200µl of 10% Ammonium Persulphate (freshly made) and 20µl of TEMED were added by order, mixed and then pour out quickly to make the gels. 6 µl of PCR product was mixed with 2µl of dye, and then loaded into gel. 6µl of low molecular weight DNA ladder (BioLabs) was used to identify the size of products The gel was run at 125V for 60 minutes in 1x TBE and then stained in 0.5µg/ml ethidium bromide solution for 30 minutes before being visualised under UV light.

2.2.4 Solutions in cloning

2.2.4.1 2.6.4 SOC medium

2g of Tryptone, 0.5g Yeast extract, 1ml 1M NaCl and 0.25 ml 1M KCl were added to 97ml dH₂O and autoclaved at 120°C for 20 min. The SOC medium was left to cool to room temperature before adding 1ml of filter-sterilised 2M Mg²⁺.

2.2.4.2 LB Agar

1g Tryptone, 0.5g Yeast extract, 0.5 NaCl and 1.5g Bacto agar were dissolved in 100ml dH₂O and autoclaved at 120°C for 20 mins. The Agar was allowed to cool for 10 min before adding 200µl 0.5µl/ml Ampicillin, 200µl 0.5µl/ml XGAL (Promega) and 0.5ml 0.1M IPTG. Mix well and pour 20ml into each plate while agar is still warm.

2.3 Immunohistochemistry

2.3.1 Tissue sectioning

All tissues for immunohistochemical analysis had been removed during surgery and formalin fixed and paraffin embedded prior to use in this study. Serial tissue sections were cut 3µm thick using a microtome.

2.3.2 *Staining*

All wash steps were with TBS and each slide was washed 3 times for a total of 5 min. Formalin fixed sections underwent antigen retrieval in Target retrieval solution (DAKO) for 40 min at 95°C, left to cool to room temperature and washed. A wax pen was used to draw around the tissue and create a well. For single stains, peroxidase blocking solution (DAKO) was applied to the tissue for 10 min and then washed. Primary antibody was diluted in antibody diluents (DAKO) at the concentration shown in the table below and the tissue was incubated for the time specified in table. At the end of the incubation time the slides were washed. Anti-Mouse/ Rabbit HRP Envision (Dako) was used as the secondary antibody and the tissue was incubated with the antibody for 30min before being washed (when primary goat antibodies were used, the tissue was first incubated with Rabbit anti-goat antibody for 30 min before the addition of mouse/rabbit HRP). The stain was developed by using DAB (Dako) for 1-5 min. Slides were then counterstained with haematoxylin before being rehydrated through water, 70% IMS, 100% IMS and twice through xylene and mounted with coverslips. Double stains were done using DAKO EnVision Double Staining System.

2.3.3 *Antibodies*

Antibody	Species	concentration	Incubation time	Antigen retrieval
CD3	mouse	1/50	1 hour	40 mins

(Dako)				
CD57 (Dako)	Mouse	1/50	1 hour	40 mins
CXCL13 (R and D AF801)	Goat	1/30	overnight	40 mins
CD21 (Dako)	mouse	1/25	1 hour	40 mins
IL-17 (Insight Biotechnology)	mouse	1/100	1 hour	40 mins
Foxp3 (abcam)	mouse	1/100	overnight	40 mins
Rabbit anti-goat (Dako)	rabbit	1/150	30 min	-

Table 2-3 Table of antibodies used in IHC

2.3.4 Cell counting in tissue sections

Cell counting in IHC stained tissue sections was done by eye using a light microscope.

The cell counts are either expressed as the number of cells per high power field or as a ratio of positive cells. For example the ratio of CD57+ cells out of all the CD3+ cells within a high power field.

2.3.4.1 Detection of cell densities in a germinal centre

All cells of interest were counted in the entire GC. The average of up to 3 different GCs was plotted for each tissue.

2.3.4.2 Detection of cell densities in a specified microenvironment.

The number of cells within an eye piece graticule, at a set magnification, was counted.

The average of three different areas counted at random was plotted for each tissue.

2.4 Flow Cytometry

2.4.1 Cell isolation

Peripheral blood mononuclear cells (PBMC) were prepared from fresh blood using Ficoll (GE Healthcare). The blood was diluted 1:1 in medium and up to 35ml of diluted blood was carefully layered over the top of 15ml of Ficoll in a 50ml falcon tube and centrifuged at 400G for 30 min with the brake and acceleration set at the lowest level or off. The PBMC layer above the Ficoll was carefully extracted with a pipette and deposited in medium. The PBMCs were centrifuged at 400G for 5 min before being resuspended in fresh medium.

Cell suspensions from tonsil and appendix were made by scrapping the tissue with a sterile surgical scalpel into fresh medium. The cell suspension was then passed through a 0.45µm cell strainer before mononuclear cells were separated using Ficoll as described above and resuspended in fresh medium.

2.4.2 Surface staining

All wash steps were performed by centrifuging at 400G for 5mins at 4°C and then pouring of the supernatant. The whole staining process was done on ice including incubation steps.

Known amount of cells were aliquoted into FACS tubes and washed in 2% FACS Buffer.

To identify T_{FH} cells the cells were incubated with antibodies to CD3, CD4, CD57, CXCR5 and PD-1. CD40L was also added to the antibody cocktail when analysing IBD patient bloods and controls in chapter 5 and CD40L expression experiment chapter 3.

T reg cells were identified using antibodies to CD14, CD4, CD127, CD25, CD45RA and PD-1.

The antibodies were diluted in 2% FACS buffer with a final volume of 100µl. The cells were incubated for 25 min in the antibody solution. The cells were washed in 2% FACS buffer and then resuspended in 400µl of 2% FACS buffer.

2.4.3 *Intracellular cytokine staining*

Tonsil mononuclear cells were cultured in for 3 hours at 37°C in a 24 well flat bottomed plate at a concentration of 2×10^6 cells/well in a total of 1ml of medium alone or medium containing 50ng/ml PMA, 750pg/ml ionomycin and Golgi Stop containing monensin (BD) (used as directed by manufacturer). The 24 well culturing plate was placed on ice for 5 min before harvesting the cells. All steps for surface staining were performed on ice. Multiple wells of stimulated cells or unstimulated cells were pooled and distributed between tests and controls. Both stimulated and unstimulated cells were washed and stained for T_{FH} cell markers as previously described for 25 min in the dark on ice and then washed with 2% FACS buffer.

Tube	Antibodies
Sample	CD3 APC-H7 CD4 Qdot 605 CXCR5PerCP Cy5.5 CD57 PB PD-1 APC
CD3,CD4 control	CD3 APC-H7 CD4 Qdot 605
Unstained cells	N/A

Table 2-4 Antibodies used to detect surface T_{FH} markers in intracellular cytokine staining experiment.

The stained cells were incubated in 1ml of 2% Paraformaldehyde for 15 min at room temperature in the dark. They were then washed with 1x biolegend Perm wash buffer. Cells were resuspended in a total of 100µl 1x biolegend Perm wash buffer, vortexed and equal amounts of cells from the sample tube were distributed between each of the three cytokine staining conditions and a surface stain only control. Condition 1 included antibodies against IL-21 and IL-4, condition 2 included antibodies against IL-17 and IFN- γ and condition 3 included antibodies against IL21 and IFN γ . Antibodies to cytokines were diluted with 1x biolegend Perm wash buffer with a final volume of 100µl and incubated on ice for 30 min. Cells were washed with 2ml 1x biolegend Perm wash buffer then 2% FACS buffer and resuspend in 200ul of 2% FACS buffer. Cells were analysed on the BD FACS Canto II 3 laser flow cytometer.

Tube	Antibodies
Unstained cells	N/A
CD3,CD4 control	CD3 APC-H7 CD4 Qdot 605
Surface stain control	CD3 APC-H7 CD4 Qdot 605 CXCR5 PerCP CY5.5 CD57 PB PD-1 APC
IL21, IL4 sample	CD3 APC-H7 CD4 Qdot 605 CXCR5 PerCP CY5.5 CD57 PB PD-1 APC IL-21 PE IL-4 FITC
IL17, IFN γ sample	CD3 APC-H7 CD4 Qdot 605 CXCR5 PerCP CY5.5 CD57 PB PD-1 APC IL-17 PE IFN- γ FITC
IL21, IFN γ sample	CD3 APC-H7 CD4 Qdot 605 CXCR5 PerCP CY5.5 CD57 PB PD-1 APC IL21 PE IFN γ FITC

Table 2-5 Antibodies used for controls and samples in intracellular cytokine staining experiment.

2.4.4 *Foxp3 staining*

In figure 4.1 tonsil mononuclear cells were surface stained antibodies against CD14-APC-H7, CD4-Qdot605, CD127-PerCP Cy5.5, both CD25-PE and PD-1-APC. In figure 5.9 PBMCs were surface stained with antibodies against CD14-APC-H7, CD4-PB, CD127-PerCP Cy5.5, both CD25-PE and CD45RA-AF488. After a 20 min incubation the cells were washed with 2% FACS buffer and the pellet pulse vortexed. The cells were resuspended in 1ml of Foxp3 1x fix/perm buffer (ebioscience) and pulse vortexed. Samples were incubated at room temperature in the dark for 45 min. 2 ml of 1x Perm buffer (ebioscience) was added to each sample and centrifuged at 400G for 5

min. The pellet was resuspended in 1x perm buffer and half transferred to a clean FACS Tube for Foxp3 FMO. Foxp3 antibody was added to the sample only and the cells were incubated at room temperature for 30 min. In figure 4.1 foxp3 PB was used, in figure 5.9, foxp3-APC was used. 2 ml of 1x Perm buffer was added to each sample before centrifuging at 400G for 5 min. 2 ml of 2%FACS buffer was added to each sample before centrifuge at 400G for 5 min. The pellet was resuspended in 2% FACS buffer and analysed on BD FACS Canto II 3 laser flow cytometer.

Tube	Antibodies
Unstained control	N/A
Treg isotype	PE isotype PercP cy5.5 isotype
CD45RA FMO	CD14 APC-C7 CD4 Pacific blue CD25 PE (M-A251) CD25 PE (2A3) CD127 PerCP Cy5.5
Foxp3 FMO	CD14 APC-C7 CD45RA AF488 CD4 Pacific blue CD25 PE (M-A251) CD25 PE (2A3) CD127 PerCP Cy5.5
Sample	CD14 APC-C7 CD45RA AF488 CD4 Pacific blue CD25 PE (M-A251) CD25 PE (2A3) CD127 PerCP Cy5.5 Foxp3 APC

Table 2-6 Antibodies used for controls and samples in foxp3 experiment in chapter 5.9

Tube	Antibodies
Unstained control	N/A
Treg isotype	PE isotype PercP cy5.5 isotype
PD-1 FMO	CD14 APC-C7 CD4 Qdot605 CD25 PE (M-A251) CD25 PE (2A3) CD127 PerCP Cy5.5
Foxp3 FMO	CD14 APC-C7 CD4 Qdot605 CD25 PE (M-A251) CD25 PE (2A3) CD127 PerCP Cy5.5 PD-1 APC
Sample	CD14 APC-C7 CD4 Qdot605 CD25 PE (M-A251) CD25 PE (2A3) CD127 PerCP Cy5.5 PD-1 APC Foxp3 Pacific Blue

Table 2-7 Antibodies used for controls and samples in foxp3 experiment in chapter 4.1

2.4.5 *CD40L time point expression analysis*

For detection of basal levels of CD40L expression tonsil lymphocytes were isolated and labelled with flow cytometry antibodies to CD3, CD4, CXCR5, CD57, PD-1 and CD40L with appropriate controls and analysed on BD FACS Canto II 3 laser flow cytometer within 4 hours of surgical removal.

Tube	Antibodies
Unstained	N/A
CD40L FMO control	CD3 APC-H7 CD4 Pacific blue CXCR5 PercP Cy5.5 CD57 FITC PD-1 APC
CD57 FMO control	CD3 APC-H7 CD4 Pacific blue CXCR5 PercP Cy5.5 PD-1 APC CD40L PE
PD-1 FMO control	CD3 APC-H7 CD4 Pacific blue CXCR5 PercP Cy5.5 CD57 FITC CD40L PE
Sample	CD3 APC-H7 CD4 Pacific blue CXCR5 PercP Cy5.5 CD57 FITC PD-1 APC CD40L PE

Table 2-8 Antibodies used for controls and samples in CD40L experiment

2 million cells per well were seeded in a 24 well flat bottomed plate in a total of 1ml of medium and incubated at 37°C for 3 hours. PMA and ionomycin were added to make a final concentration of 50ng/ml and 750pg/ml in consecutive wells at time 0, 1h, 2h, 2h30min and 2h45min so that cells were stimulated for 3h, 2h, 1h, 30min and 15min. The plate was put on ice for 10min to detach adherent cells before transferring the cells from each well into separate FACS tubes. Each tube was washed with 2%FACS buffer and cells labelled with antibodies CD3-APC-H7, CD4-PB, CD57-FITC, CXCR5-PerCP Cy5.5, PD-1-APC and CD40L-PE for 25 min. The cells were washed and resuspended in 500µl of 2%FACS buffer before being analysed on BD FACS Canto II 3 laser flow cytometer.

2.4.6 CFSE labelling and culturing experiment

Tonsil cell suspensions were labelled with antibodies CD3-APC-H7, CD4-Qdot605, CD57-PB, CXCR5-PerCP Cy5.5 and PD-1-APC. PD-1⁺ CD57⁺ and PD-1⁺ CD57⁻ T_{FH} cell subsets were sorted by FACS Aria flow cytometer (BD) into RPMI 10% FCS 1% Pen-strep.

Tube	Antibodies
Unstained	N/A
CXCR5 FMO control	CD3 APC-H7 CD4 Qdot605 CD57 Pacific blue PD-1 APC
CD57 FMO control	CD3 APC-H7 CD4 Qdot605 CXCR5 PE PD-1 APC
PD-1 FMO control	CD3 APC-H7 CD4 Qdot605 CXCR5 PE CD57 Pacific blue
Sample	CD3 APC-H7 CD4 Qdot605 CXCR5 PE CD57 Pacific blue PD-1 APC

Table 2-9 Antibodies used for controls and samples when sorting T_{FH} subsets in CFSE labelling and culturing experiment

Sorted cells were washed twice in 1x PBS and the pellet was dislodged. 1ul/ml CFSE in PBS was freshly made and 250ul of 1ul/ml CFSE was added to each pellet of sorted cells and incubated at 37°C for 7 min and shaken once half way through. 250µl of FCS was added to each tube to quench the CFSE and each tube was topped up with 2 ml of medium. The cells were washed and resuspended the in sterile medium. In a sterile 96 round bottomed plate 1x10⁵ unlabelled autologous tonsil cells were added to the mixed culture wells and unlabelled control wells. Equal numbers of CFSE cells were added to wells containing unlabelled and unsorted whole tonsil mononuclear cell suspension and wells with media alone. The cells were cultured for 4 days at 37°C and

FACS all the wells separately for CD57-PB and 7AAD before analysis on BD FACS canto II 3 laser and flowjo.

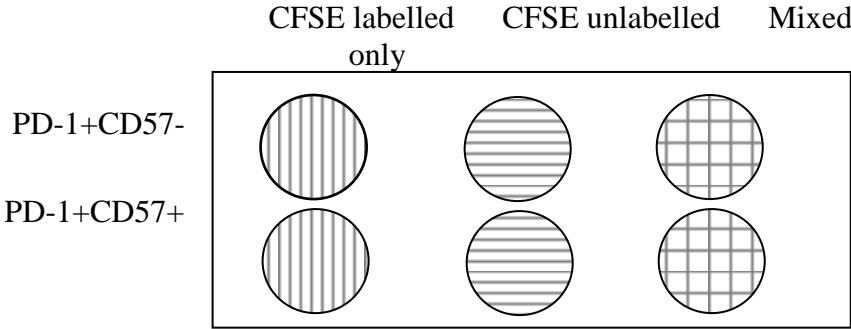


Figure 2-1 A diagram of the set up and contents of the culturing plate in CFSE and culturing experiment.

2.4.7 FACS Antibodies

Antigen	Fluorochrome	Company	Clone
CD3	APC-H7	BD	SK7
CD4	Pacific Blue	Biolegend	RPA-T4
CD4	Qdot 605	Invitrogen	S3.5
CD14	APC-cy7	Biolegend	HCD14
CXCR5	PE	R and D	51505.11
CD185 (CXCR5)	PerCP Cy5.5	Biolegend	TG2
CD57	FITC	BD	NK-1
CD57	Pacific Blue	Biolegend	HC57
CD279 (PD-1)	APC	BD	MH4
CD127	PerCP Cy5.5	eBioscience	BioRDR
CD25	PE	BD	2A3
CD25	PE	BD	M-A251
CD45RA	Alexa fluor488	Biolegend	HI100
CD154 (CD40L)	PE	eBioscience	24-31
IL-21	PE	Biolegend	3A3-N2
IL-4	FITC	Biolegend	MP4-25D2
IL-17	PE	Biolegend	BC168
IFN- γ	FITC	Biolegend	B27
Foxp3	Alexa fluor 647	BD	259D/C7
Foxp3	Pacific Blue	Biolegend	206
7AAD	-	BD	

Mouse isotype IgG1κ	PE	BD	MOPC-21
Mouse isotype IgG1κ	PerCP-Cy5.5	eBioscience	P3.6.2.8.1
Mouse isotype IgG1κ	APC	BD	MOPC-21

Table 2-10 All antibodies used for flow cytometry

2.5 Nucleotide extraction and PCR

All nucleotide extraction and PCR preparation was carried out in UV sterilised hoods in specially designated rooms. PCR master mix preparation was performed in separate rooms to nucleotide extraction to prevent contamination of primers and buffers. All equipment such as pipettes, plates and tubes were UV sterilised before use. Nuclease free water was used in all PCR experiments and aliquots were UV sterilised before each use.

2.5.1 DNA extraction

DNA was extracted from sorted cells using a DNA extraction kit (Qiagen) according to manufactures instructions.

DNA was stored at -20°C.

2.5.2 RNA extraction

RNA was extracted from sorted cells using the RNeasy Mini kit (Qiagen) according to manufactures instructions.

The RNA was stored at -80°C.

2.5.3 *Reverse Transcription*

14µl extracted RNA, 6µl H₂O and 2µl OligoDT were added to a sterile PCR tube on ice and vortex before a hot start for 10min at 95°C. The PCR tubes were immediately placed back on ice for 5 min, vortexed and replaced back on ice for a further 5 min. 8µl M-MLV buffer, 6µl H₂O, 2µl RT reverse transcriptase, 1µl RNase inhibitor and 1µl dNTPs was added to the RNA solution on ice. The PCR tubes were placed in the PCR machine and run at 42°C for 60min and 70°C for 15 min. The resulting cDNA was then stored at -20°C.

2.5.4 *Real-time PCR*

In a 384 well plate 5.5ul TaqMan Gene expression master mix (Applied Biosystems), 0.5ul primer and 4ul cDNA were add per well and run on the standard setting on 7900HT sequence detection system (Applied Biosystems). Each sample was run in duplicate for both the house keeping gene and the target gene. The house keeping gene used in this study was GAPDH.

GAPDH (Applied biosystems) Hs02758991_g1

Bcl-6 (Applied biosystems) Hs00153368uml

2.5.5 *TCRγ PCR*

The TCRγ chain was amplified by PCR using Vγ11, Vγ101 and Jγ11 primers. Each reaction consisted of 30µl nuclease free H₂O, 10µl 5x flexi buffer, 3µl MgCl₂, 0.5µl 20mm dNTPs, 0.5µl 20mm of each primer and 3µl DNA. 2µl of taq polymerase was added after the hot start. After a hot start of 7min at 95°C ,40 cycles of 93°C for 1 min, 55°C for 1 min and 73°C for 1 min were performed, followed by a final extension time of 7 min at 73°C. PCR products were visualised on a 10% mini polyacrylamide gel.

2.5.6 *TCR β PCR*

The TCR β chain from the T cell subsets was amplified by a semi nested PCR using primer to the V03 and J1.5 regions. Each first round reaction consisted of 23.5 μ l nuclease free H₂O, 10 μ l 5x flexi buffer, 3 μ l MgCl₂, 0.5 μ l 20mm dNTPs, 0.5 μ l 20mm of V03 external primer and J1.5 primer and 10 μ l DNA. After a hot start of 7 min at 93°C, 30 cycles of 95°C for 1 min, 50°C for 1 min and 73°C for 1 min 30 sec were performed, followed by a final extension time of 10 min at 73°C. 3 μ l of the first round product was added to the second round PCR reaction which contained of 30.5 μ l nuclease free H₂O, 10 μ l 5x flexi buffer, 3 μ l MgCl₂, 0.5 μ l 20mm dNTPs, 0.5 μ l 20mm of V03 external primer and J1.5 primer and 2 μ l of taq polymerase was added after the hot start. The PCR program was the same as the first round. PCR products were visualised on an agarose gel.

2.5.7 *Primers*

V γ 11 primer 5' TCTGGRGTCTATTACTGTGC 3'

V γ 101 primer 5' CTCACACTCYCACTTC 3'

J γ 11 primer 5'CAAGTGTTGTTCCACTGCC 3'

V β 03 External primer 5'CCCAGACTCCAAAATACCTGG 3'

V β 03 Internal primer 5'CCTGGTCACACAGASGGG 3'

J β 1.1 primer 5' TGAGTCTGGTGCCTTGTCC 3'

J β 1.5 primer 5' AGAGTCGAGTCCCATCACC 3'

M13 Forward 5' GTAAAACGACGGCCAGT 3'

M13 Reverse 5' GGAAACAGCTATGACCAGT 3'

2.6 Cloning of PCR products

2.6.1 Purification of PCR products

PCR products to be used in cloning were purified with MiniElute PCR purification Kit (Qiagen) according to the manufacturers protocols and stored at -20 °C until use.

2.6.2 Ligation

The pGEM-T Vector Kit (Promega) was used to ligate the PCR products. The ligation reaction contained 5µl buffer, 3µl purified PCR product, 1µl T4 DNA ligase, 1 µl vector and these were mixed together on ice. The ligation reaction was then incubated at room temperature for 2 hours.

2.6.3 Transformation

2.5µl of ligation reaction was added to 25µl of bacteria (JM109 competent cell, Promega) in a sterile 1.5ml centrifuge tube and incubated on ice for 20 minutes. The vector was inserted into the bacteria by heat shock in a water bath at 42°C for 45 seconds and then placed immediately back on ice for 2 minutes. 950µl of cold SOC medium was added and incubated at 37°C, shaking at 150rpm for 2 hours. To collect the bacteria the solution was centrifuged at 200rpm for 10 minutes at room temperature. The supernatant was discarded and the bacteria pellet was resuspended in 100µl of cold SOC medium. All 100µl of bacterial suspension was spread onto an LB Agar plate. The plates were inverted and incubated over night at 37°C.

2.6.4 Picking up colonies

Bacteria with a vector containing a DNA insert grew as white colonies and the bacteria with a vector without a DNA insert grew as blue colonies. The white colonies were picked up at random with a pipette tip and transferred to a gridded plate and incubated at 37°C overnight. The remaining bacteria on the tip was washed off in 15µl

dH₂O. The samples were boiled for 10 min to release the vector and denature the DNase and then centrifuged at 400 G for 10 min. Samples were stored at -20°C until use.

2.6.5 *Screening clones by PCR*

5µl of bacterial supernatant was amplified by PCR using M13 forward and M13 reverse primers. One PCR reaction contained 9.46µl nuclease free dH₂O, 4µl 5x flexi buffer, 1.2µl MgCL₂, 0.2µl 20mm dNTPs, 0.02µl of each 100mm M13 primers, 0.1µl taq polymerase. PCR products were run on a 3% agarose gel to screen for inserts of the correct size.

2.6.6 *DNA sequencing*

200µl of agar containing ampicillin only was added to each well in 96 round or flate bottomed culture plate and left to cool and set. Colonies with appropriately sized DNA inserts were stabbed and transferred to a well in the 96 well plate. The plate was sealed with a sticker lid and bound with paraffin tap and sent for template prep and sequencing by Beckman Coulter sequencing services.

2.7 *Sequence analysis*

Using Gene Jockey sequence analysis program, the V and J regions were identified and primers and vector sequences were removed. Cut sequences were aligned and compared. Identical sequences originating from the same PCR were considered to be possibly the result of PCR amplification from one cell and considered as representing one cell. Sequences with the same J and V genes and the exact same nucleotides in the N region from different PCRs were considered to be derived from clonally related cells.

2.8 Statistics

Statistically significant differences between groups of data are indicated by a bar over the data. Data sets that are not significantly different to each other have been left blank. Statistical tests were carried out using GraphPad Prism 4. Gaussian or non-Gaussian distribution was established by D'Agostino and Pearson omnibus normality test. Unpaired data with Gaussian distribution were analysed by an unpaired t test. Unpaired data with a non-Gaussian distribution were analysed by a Mann-Whitney test. Paired data with Gaussian distribution were analysed by a paired t test. Paired data with non-Gaussian distribution were analysed by a Wilcoxon matched pairs test. Populations were considered to be different to each other if $P \leq 0.05$.

3 Chapter 3: T_{FH} cells in Peripheral and Gut Associated Lymphoid Tissue

3.1 Introduction

The plasma cells of the intestinal humoral immune system are largely located in the intestinal LP throughout the small bowel and colon, though some are known to reside in the bone marrow. In contrast, the long-lived plasma cells of the peripheral immune system, most of which secrete IgG, are largely restricted to the bone marrow (Mei et al., 2009). Immunoglobulins secreted by plasma cells in these different locations have different features in their antigen binding profile. Recent studies that construct human monoclonal antibody secreting cell lines by engineering the sequences encoding the used heavy and light chains of isolated single cells into expression systems, have demonstrated that approximately 26% of the IgA and IgG secreting LP plasma cells are polyreactive to two or more antigens, including self-antigens (Benckert et al., 2011). Although autoreactive and polyreactive antibodies circulate in the blood of healthy individuals (Dighiero et al., 1986), the antibodies secreted by human IgG bone marrow plasma cells tend to have a lower frequency of autoreactive or polyspecificity than those in the gut.

The differences in specificity in the intestinal and bone marrow plasma cell populations are likely to reflect the properties of the different lymphoid tissue that generate them. Whereas the IgA response is driven by the diverse and abundant microbial flora in the gut lumen, the bone marrow IgG plasma cells are derived from affinity matured responses to antigen encountered systemically (Brandtzaeg et al., 1999). B cells activated in GALT become plasmablasts that enter the blood via the lymphatic system. They then mostly home back to the intestine by a system mediated by the expression of chemokine receptors CCR9, CCR10 and the integrin $\alpha 4\beta 7$ where they

differentiate into plasma cells in the LP (Pabst et al., 2004, Hieshima et al., 2004, Farstad et al., 1995). In contrast IgG plasma cells home to the bone marrow by chemokine receptors including CXCR4 (Kunkel and Butcher, 2003). The majority of the plasma cells that home to the gut LP secrete dimeric IgA that binds to the polymeric immunoglobulin receptor (pIgR) and is actively transported across the epithelial barrier into the intestinal lumen (Brandtzaeg, 1974). This compartmentalisation of the IgA system which results in IgA secretion to the outside of the body may explain why polyspecificity and autoreactivity can be tolerated in the IgA system but not by IgG produced in the bone marrow that provides a long-lived protection in serum and body fluids that could be proinflammatory and potentially pathogenic if not tightly regulated.

The IgV genes used by human intestinal plasma cells are almost all mutated by somatic hypermutation suggesting that they are derived from germinal centre responses in GALT (Barone et al., 2011, Dunn-Walters et al., 1997). Such diversity in the IgA repertoire has been shown to be essential for intestinal health (Wei et al., 2011). The presence of IgV gene mutations in polyreactive and autoreactive mucosal immunoglobulins suggests that these plasma cells were also generated through a GC reaction (Benckert et al., 2011). Although there is good evidence that IgA responses can be T cell independent and GC independent, the prevalence of T cell independence in the human IgA system is not understood, but the vast majority of plasma cells appear to be germinal centre derived (Bergqvist et al., 2006, Bergqvist et al., 2010).

The process of affinity maturation in GCs is regulated in part by T_{FH} . T_{FH} aid the selection and maturation of clonally expanded B cells within the GC. Newly formed centrocytes in the GC present antigen to the T_{FH} via cognate interactions and the T_{FH} provides survival signals through CD40L and cytokines to the centrocytes that have higher affinity for the antigen (Breitfeld et al., 2000, Bryant et al., 2007). Production of

cytokines including IL-21, IL4 and IFN γ by T_{FH} have been reported (Luthje et al., 2012). A selective pressure favouring the development of plasma cells secreting high affinity antibodies is in part controlled by restricting the number of T_{FH} (Linterman et al., 2011). In mice it has been demonstrated that an uncontrolled increase in T_{FH} leads to systemic autoimmunity (Linterman et al., 2009).

T_{FH} are themselves thought to be associated with several different pathways of T cell development. Although T_{FH} in humans express the antigen CD57 that is associated with end-stage differentiation, T_{FH} in mice have recently been shown to be able to differentiate along multiple effector T cell pathways (Luthje et al., 2012). T_{FH} are regulated by subsets of regulatory CD4 and CD8 T cells. Approximately 10% of human and murine T_{FH} have regulatory properties and express foxp3 and are termed follicular regulatory T cells (Tfr) (Linterman et al., 2011, Lim et al., 2005). In mice it is proposed that a regulatory subset that resides within the CD8 T cell compartment regulates T_{FH} to maintain self-tolerance (Kim et al., 2010). It has been suggested that a reduction in follicular regulatory cells leads to an increase in T_{FH}, an increase in output of non-specific and a reduction in the antigen specific GC response (Linterman et al., 2011).

PD-1 is highly expressed by T_{FH} and expression of PD-1 by T_{FH} has been associated with the development of high affinity B cells in GCs (Good-Jacobson et al., 2010). Signalling through PD-1 negatively regulates T cell proliferation and cytokine production (Freeman et al., 2000). Disruption of PD-L1 expression or blockade of PD-L1 or PD-1 lead to an increase in T_{FH}, an increase in antibody production, and development of autoimmune disease (Hams et al., 2011, Nishimura et al., 1999). Data from a PD-1 deficient mouse model has demonstrated that PD-1 regulates the specificity of IgA responses and hence the integrity of the mucosal barrier (Kawamoto et al., 2012). In PD-1 deficiency the number and nature of T_{FH} are altered (Kawamoto et

al., 2012). PD-1 expression is therefore interesting in the context of B cell selection in GCs from different anatomical locations in humans, and of particular relevance to the regulation of the IgA plasma cell population.

The relatively high frequency of poly and autoreactive plasmablasts found in the gut suggests that selection for antigen specificity may be less stringent in GALT GCs compared to those in peripheral lymphoid tissues. Investigations reported in this chapter aim to determine if there is evidence for differences in T_{FH} in the gut versus systemic lymphoid tissue that may be involved in regulating the stringency of humoral responses differently in these sites.

- To determine if there is any difference in the number or phenotypic diversity of T_{FH} in the germinal centres of GALT versus peripheral lymphoid tissues that might reflect a role of T_{FH} in determining the functional profile of mucosal immunoglobulins.
- To determine whether there is any difference in functional parameters of subsets of T_{FH} that could be related specifically to germinal centre function.
- To determine if subsets of T_{FH} might be developmentally related.
- To determine if there is any difference in regulatory factors which effect T_{FH} frequency.

3.2 Results

3.2.1 *Phenotypic diversity of T_{FH} in germinal centres of GALT vs. peripheral lymphoid tissues*

3.2.1.1 *Frequency of CD57+ T_{FH} in GALT and peripheral lymphoid tissues.*

In lymphoid tissue sections, T_{FH} can easily be identified as the T cells within the GC microenvironment. To explore the frequency of T_{FH} , tissue sections of different human

lymphoid tissues were double stained for CD3 and CD57. T_{FH} were identified as all cells positive for CD57/CD3 or CD3 alone within the GC (Fig 3.1A).

From counting the CD57⁺ and CD3⁺ only cells within the GC, the percentage of CD57⁺ T_{FH} was generated as a number of CD57⁺ cells/ number of CD57⁺ cells + number of CD3⁺ only cells. On average 92% of peripheral lymphoid tissue (tonsil and spleen) T_{FH} were CD57⁺ (Fig 3.1C) and the percentage of CD57⁺ T_{FH} cells was consistent between tonsil and spleen (Fig 3.1B). However, a lower percentage of T_{FH} were CD57⁺ in GALT (ileum, appendix and colon), where on average around 60% of T_{FH} were CD57⁺ (Fig 3.1C). The percentage of CD57⁺ T_{FH} was consistently lower throughout the gut compared to peripheral lymphoid tissues (Fig 3.1B). Therefore two T_{FH} subsets have been identified which can be distinguished by their expression of CD57 and a greater proportion of T_{FH} express CD57⁺ T_{FH} in peripheral lymphoid tissue compare to GALT.

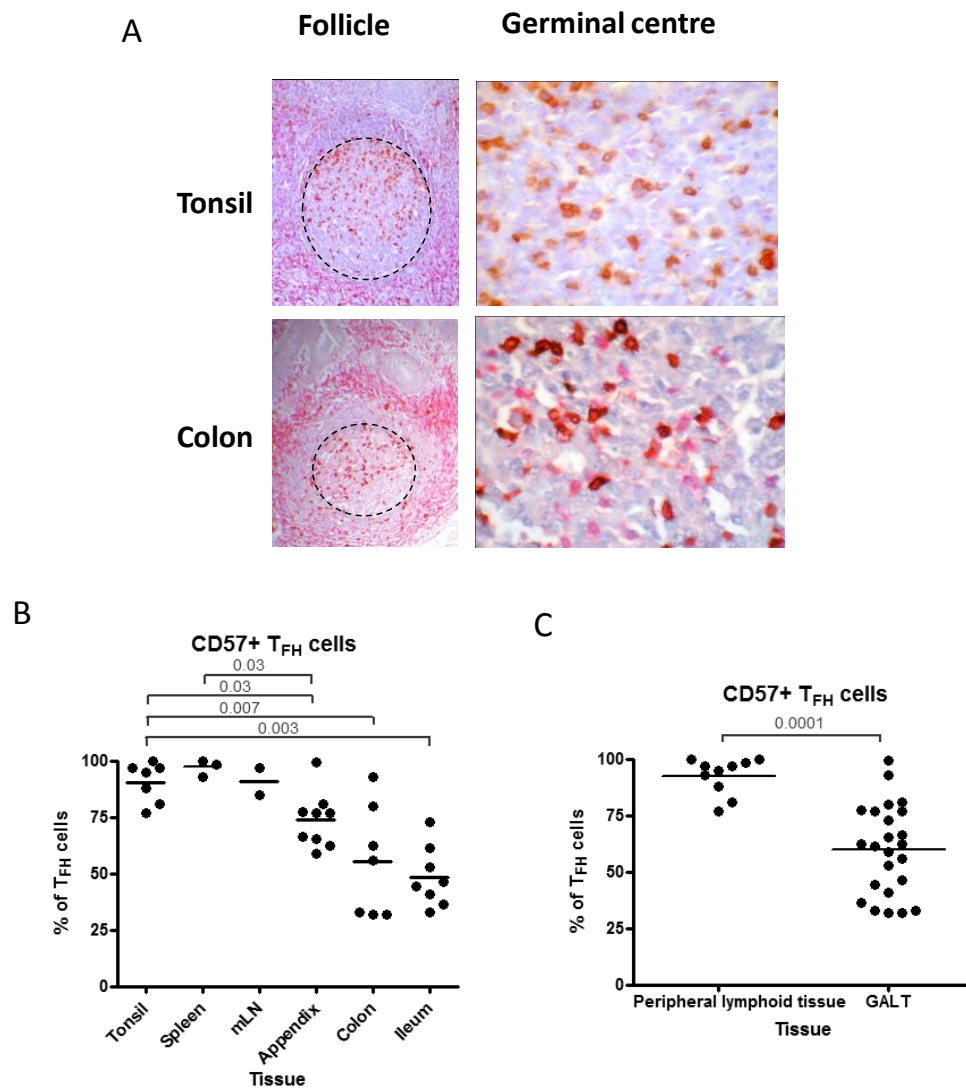


Figure 3-1 Frequency of CD57+ and CD57- T_{FH} within GCs of secondary lymphoid tissues

A- Examples of tissue sections of tonsil and colon stained for CD57 (brown) and CD3 (pink). Left GC x40 and right GC x100 magnification. B- Average percentage of CD57+ T_{FH} found within 1, 2 or 3 GCs of tonsil (n=7), spleen (n=3), mLN (n=2), appendix (n=8), colon (n=7) and ileum (n=8). C - Average percentages of CD57+ T_{FH} within GC of peripheral lymphoid tissues (tonsil and spleen) and GALT (ileum, colon and appendix). Mann Whitney statistical test performed on B and C.

3.2.1.2 Relative T_{FH} density in GALT and peripheral lymphoid tissues.

With the identification of two subsets of T_{FH} which differ in proportion in GALT and peripheral tissues, the ratio of T_{FH} subsets to other GC cells was investigated. By using tissue sections double stained for CD57 and CD3, the number of CD57+, CD3+ and unstained but haematoxylin positive cells (non T cells), such as B cells, follicular dendritic cells and stromal cells, within GCs were counted. This was then used to generate ratios of T_{FH} cells to non T cells as a measure of relative T_{FH} density.

The relative density of total T_{FH} was significantly higher in appendix and ileum compared to tonsil (Fig 3.2A). Overall GALT had a higher average relative T_{FH} density (0.47) than in peripheral lymphoid tissues (0.27) (Fig 3.2D).

A significantly higher relative density of CD57- T_{FH} is observed in GALT compared to peripheral lymphoid tissue, however, the relative density of CD57+ T_{FH} was comparable (Fig 3.2E and F). The relative density of CD57- T_{FH} is consistent higher throughout GALT compared to tonsil (Fig 3.2C). Thus, the higher frequency of CD57- T_{FH} is probably responsible for the higher frequency of T_{FH} in GALT compared to peripheral lymphoid tissues.

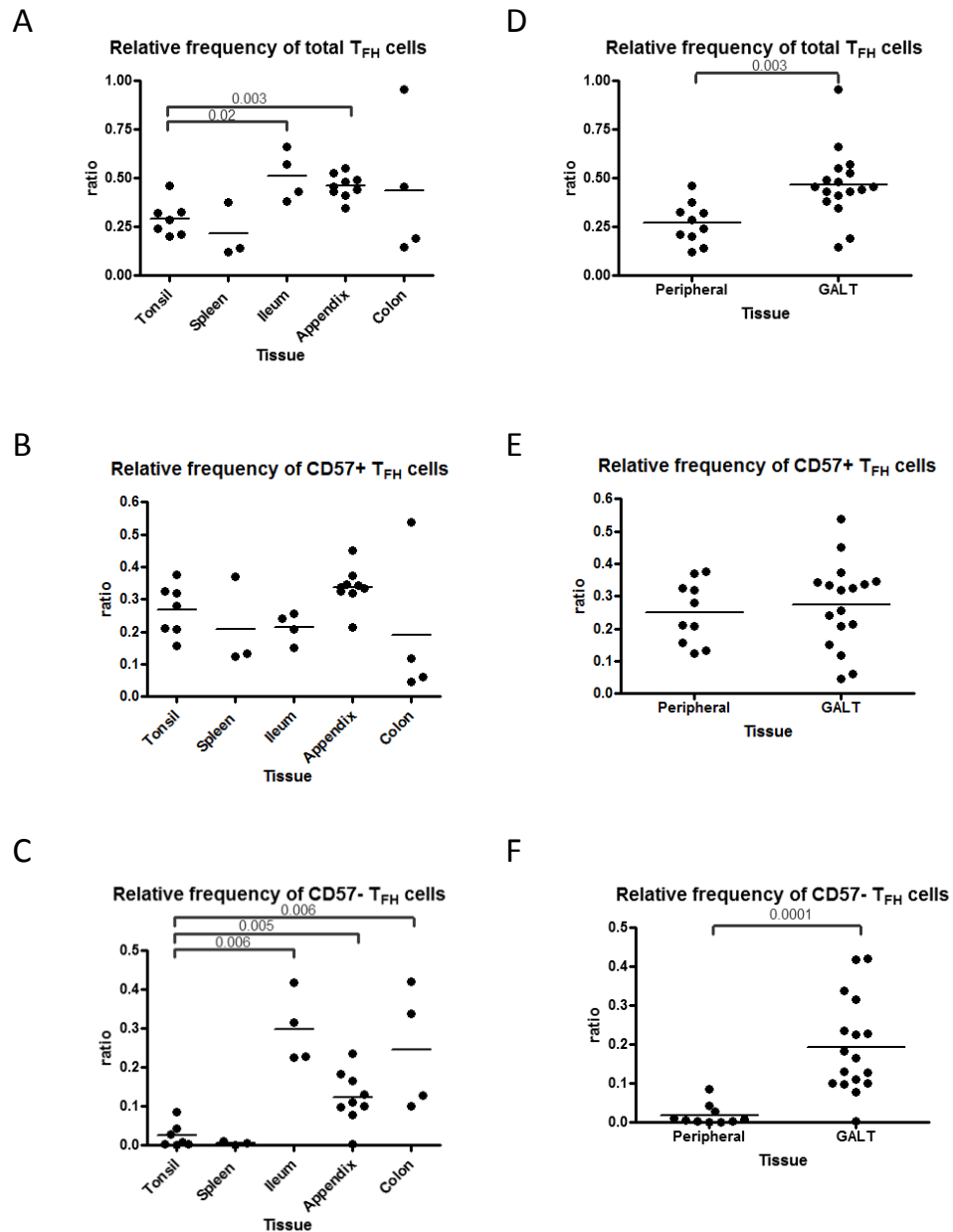


Figure 3-2 Ratio of T_{FH} subsets to other non T cells within peripheral and GALT GCs.

Ratio generated by comparing the total number of $CD3^+$, $CD57^+$ or both to the number of haematoxylin only stained cells within GCs. >3 GCs were counted and the average ratio plotted. A-C Relative frequency of T_{FH} subsets within GCs of tonsil ($n=7$), spleen ($n=3$), ileum ($n=4$), appendix ($n=9$) and colon ($n=4$). D-F Relative frequency of T_{FH} within GC of peripheral lymphoid tissues (tonsil and spleen) and GALT (ileum, colon and appendix). A and D- Relative frequency of total T cells to all other

cells within GCs. B and E- Relative frequency of CD57+ T cells to all other cells within GCs. C and F Relative frequency of CD57- cells to all other cells within GCs. Mann-Whitney statistical test performed on A-C and F. Unpaired T test performed on D and E.

To explore the positioning of the two subsets of T_{FH} within the GC, the relative density of total T cells, CD57+ T cells and CD57- T cells to non T cells was explored in the light and dark zones of the GC (Fig 3.3A). Tonsil tissue was used as an example of peripheral lymphoid tissue and appendix as an example of GALT. As the GC in the tissue section has to be in the right orientation to determine these relative densities, a smaller data set was obtained.

CD57- T cells were present in both the light zone and dark zone of appendix GCs (Fig 3.3D and G). However, there was no significant difference in the relative density of T_{FH} subsets in either the light or dark zone of the GC between tonsil and appendix tissues (Fig 3.3 C, D F, and G).

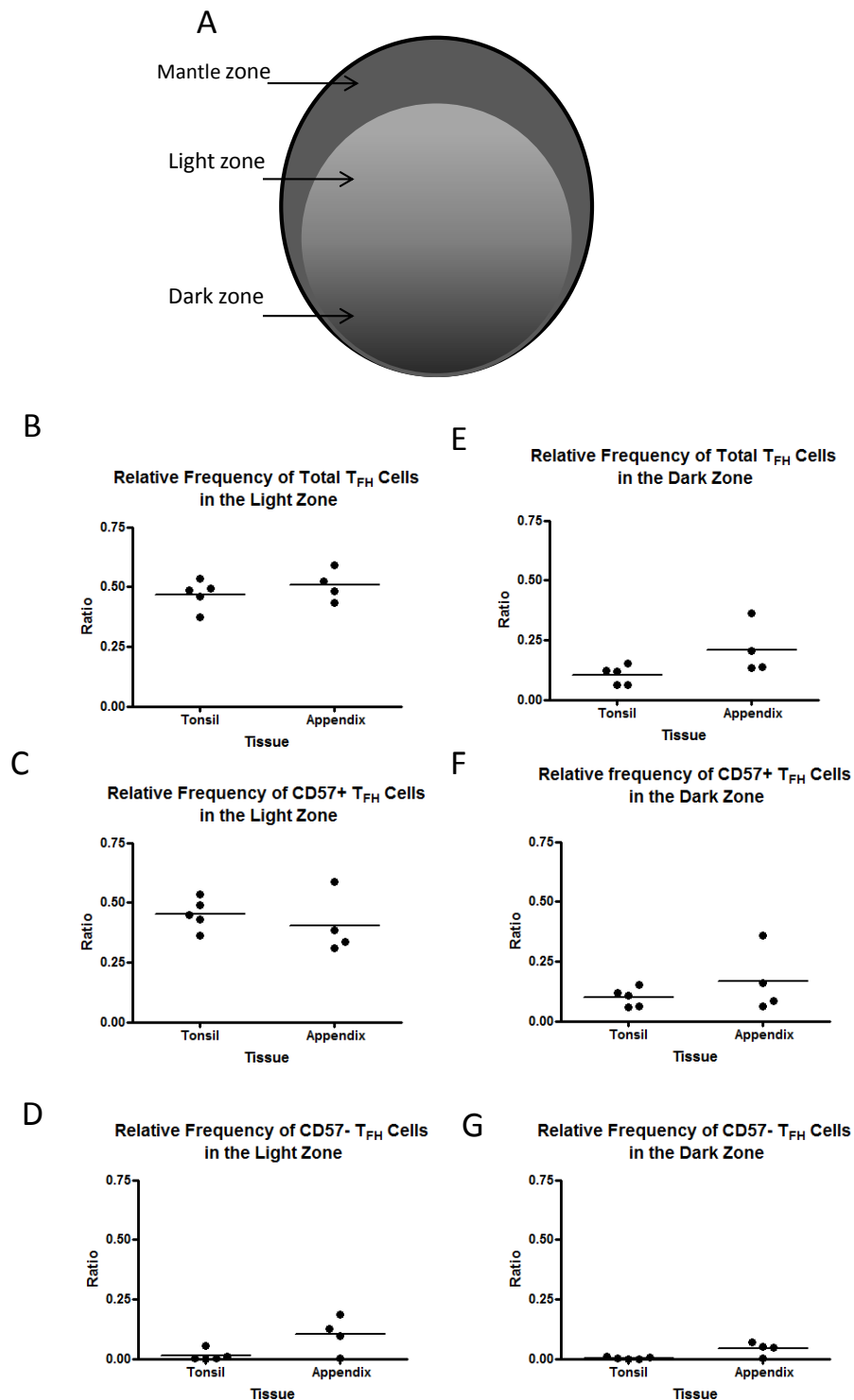


Figure 3-3 Ratio of T_{FH} with in the light and dark zone of GCs

A-Diagram of a germinal centre depicting the light and dark zones. A-G Average ratio of 1, 2 or 3 GCs from tonsil (n=5) and appendix (n=4). B and E- Relative frequency of T cells to non T cells in either the light or dark zone. C and F- Relative frequency of $CD57^+$ T cells to non T cells in either the light or

dark zone. D and G- Relative frequency of CD57- T cells to non T cells in either light or dark zone. Mann Whitney statistical test performed on B-G.

3.2.2 *Defining T_{FH} subsets in lymphoid tissue cell suspension by flow cytometry*

In order to further analyse T_{FH} in GALT and peripheral lymphoid tissue, fresh T_{FH} from lymphoid tissues were isolated. A panel of flow cytometry antibodies were designed to identify T_{FH} as accurately possible and the detection of the T_{FH} transcription factor, Bcl-6, was used to investigate T_{FH} cell status.

Cells within the $CXCR5^{high}$ population were separated by their expression of CD57 and PD-1. Three distinct populations of cells were identified within the $CXCR5^{high}$ CD4 T cell gate; PD-1^{low} CD57-, PD-1^{high}CD57- and PD-1^{high}CD57+. A very low frequency of PD-1^{low}CD57+ cells were also identified (Fig 3.4A). The subsets within the $CXCR5^{high}$ CD4 T cell gate were isolated by flow cytometry and RT-PCR was used to detect the presence of Bcl-6 mRNA. The two subsets which expressed the highest levels of Bcl-6 were PD-1^{high} in tonsil tissue (Fig 3.4B). So few PD-1^{low}CD57+ cells were isolated that Bcl-6 and the house keeping gene were both undetected in this subset and therefore cannot be ruled out as a T_{FH} subset. However from the data that was obtained, cells that were CD3+CD4+ $CXCR5^{high}$ PD-1^{high} (highlighted in red box in Fig 3.4A) were considered T_{FH} and this is consistent with other studies (Simpson et al., 2010). T_{FH} identified in this way were then divided into CD57+ and CD57- subsets.

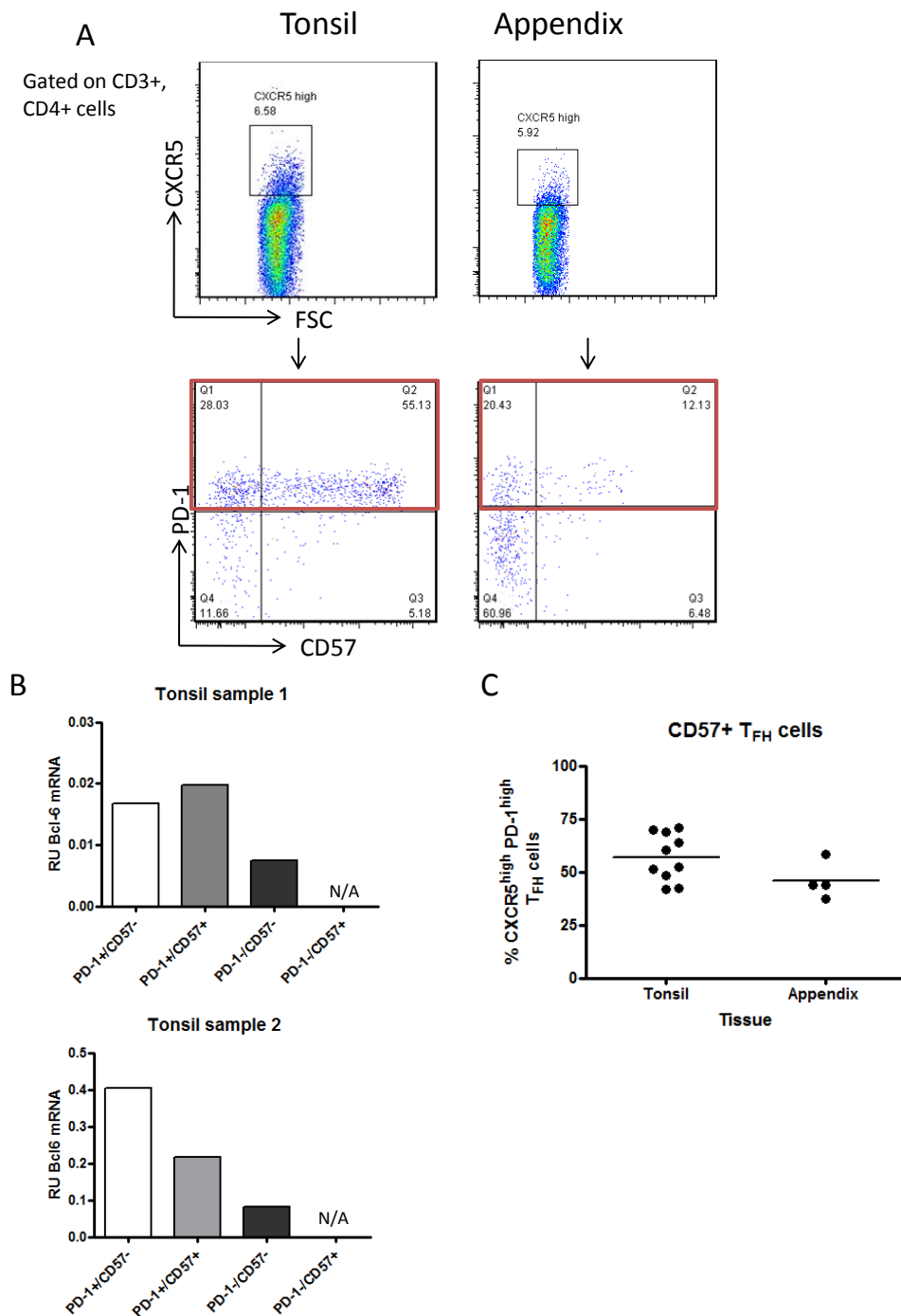


Figure 3-4 Identifying T_{FH} subsets within the CXCR5^{high} CD4 T cell population in lymphoid tissue cell suspension.

Tonsil mononuclear cells were stained with flow cytometry antibodies to CD3, CD4, CXCR5, PD-1 and CD57. A- Example of gating on CXCR5^{high} previously gated on CD3+, CD4+ cells (top FACS plots) and the subsets of cells within this gate separated by their expression of PD-1 and CD57 (bottom FACS plots) in tonsil and appendix cell suspension. B- CXCR5^{high} CD4 T cell subsets as defined in bottom left FACS plot in A were sorted by FACS and bcl-6 expression was determined by real-time PCR in two

example of tonsil C- FACS analysis of the percentage of CD57+ T_{FH} within the CXCR5^{high}PD-1^{high} T_{FH} gate in tonsil (n=10) and appendix (n=4) fresh mononuclear cell suspension. Mann-Whitney statistical test performed on C.

The relative frequencies of T_{FH} expressing CD57 was analysed in fresh tonsil and appendix cell suspensions by using the method of gating to identify T_{FH} illustrated in figure 3.4A. The relative frequency of CD57 expressing cells in the CXCR5^{high}PD-1^{high} population was not significantly different between tonsil and appendix when analysing by flow cytometry though a trend towards a higher frequency of CD57+ T_{FH} in tonsil can be seen (Fig 3.4C). As a significant difference in the percentage of CD57+ T_{FH} was seen between GALT and peripheral lymphoid tissues using IHC methods in figure 3.1, this finding is probably a result of the methods used to detect T_{FH} and will be discussed below.

3.2.3 *Differences in functional factors expressed by T_{FH} subsets*

To investigate the functional significance of the relatively high frequency of CD57- T_{FH} in GALT, functional parameters were explored.

3.2.3.1 *T_{FH} subsets and expression of cytokines*

To further characterize the CD57+ and CD57- T_{FH} subsets the cytokine profiles were investigated. Mononuclear cells from tonsil single cell suspension were stimulated for 3 hours with PMA and ionomycin. T_{FH} were identified by flow cytometry as being CD3+CD4+CXCR5^{high}PD-1+ and subsets of T_{FH} were separated by their expression of CD57 (Fig 3.5A). For each subset the frequency of cells producing IL-21 and IL4 was determined by intracellular FACS analysis (Fig 3.5A). Both subsets of T_{FH} produced IL-21 and IL-4 however a significantly higher proportion of single IL-21+ cells and total IL-21+ cells were observed within the CD57- T_{FH} subset compared to the CD57+ T_{FH} subset (Fig 3.5B).

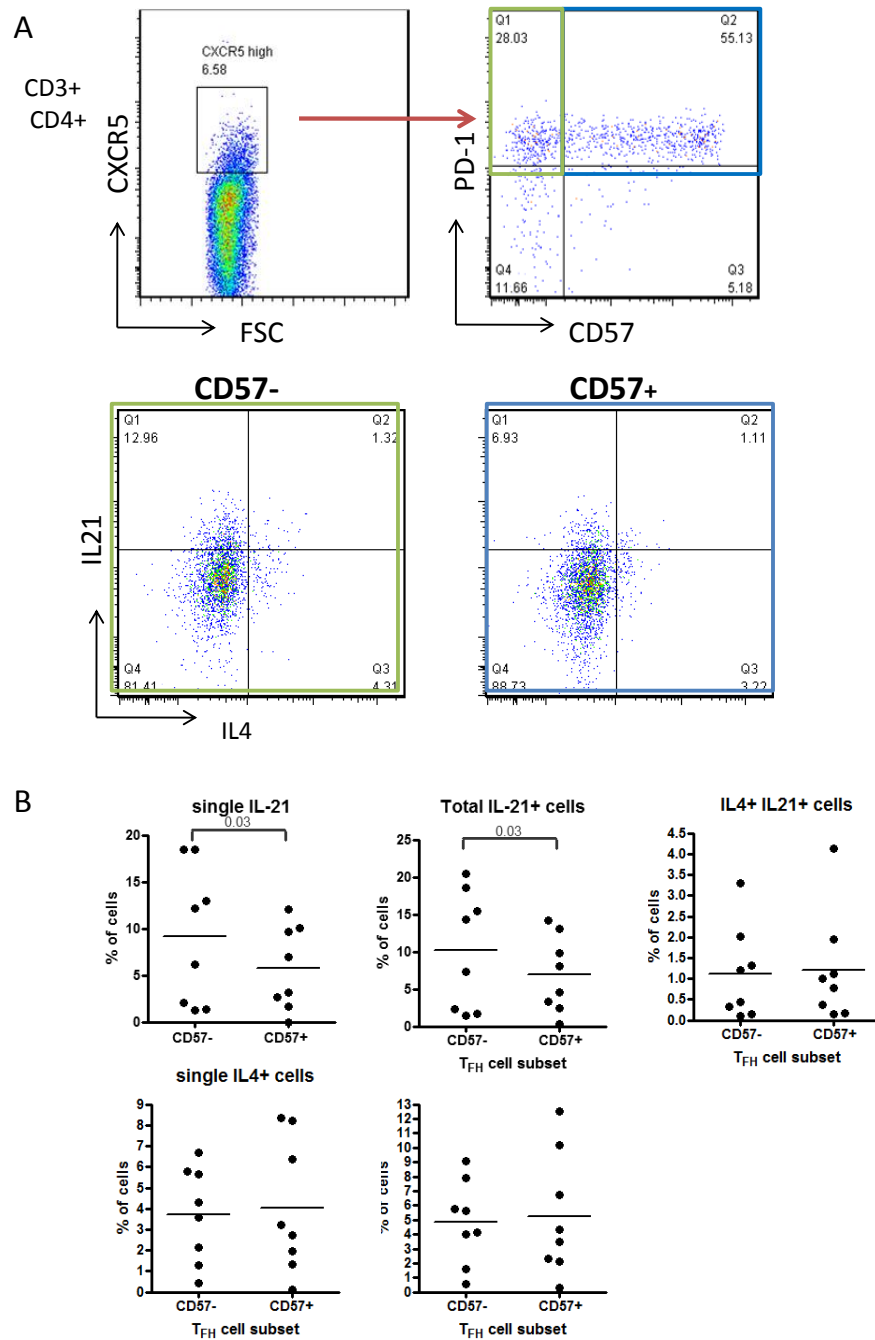


Figure 3-5 T_{FH} associated cytokine production by T_{FH} subsets

Tonsil (n=7) mononuclear cells stimulated with PMA and ionomycin for 3 hours and T_{FH} subsets assessed by flow cytometry for IL-21 and IL-4 production A-An example of the gating strategy to identify T_{FH} by flow cytometry in a tonsil and the separation of IL21+ and IL4+ cells within the CD57+ and CD57- T_{FH} gates. B- The percentage of CD57+ and CD57- T_{FH} single positive for IL-21 or IL-4, total IL-21+ or IL-4+ and IL-4+IL-21+ cells. Wilcoxon matched pairs statistical test performed on B.

T_{FH} have been reported to express cytokines that are more typically associated with other T cell subsets. IFN γ production is normally associated with a Th1 phenotype. However, intracellular cytokine analysis of IL-21 and IFN γ double positive cells showed that a small percentage of CD57+ and CD57-T_{FH} expressed both IL-21 and IFN γ (Fig 3.6 A).

IL-17+ is a proinflammatory cytokine which is most commonly associated with Th17 cells. Analysis of IL-17 and IFN γ production by T_{FH} subsets by flow cytometry showed that IL-17+ cells were present in both CD57+ and CD57- T_{FH} populations along with IFN γ . Analysis of both single and total IL-17+ cells showed a significantly higher percentage of IL-17+ cells within the CD57- T_{FH} subset compared to the CD57+ T_{FH} subset (Fig 3.6B).

Further characterisation of IL-17 expression in tissue sections of tonsil and appendix identified IL-17 secreting cells within the GC microenvironment (Fig 3.6C). This is consistent with the identification of IL-17+ cells within the T_{FH} compartment in figure 3.6B. However IL-17+ cells are more frequent within the lymphocyte area compared to the GC in both tonsil and appendix tissue, probably due to the overall higher frequency of T cells compared to GCs (Fig 3.6C).

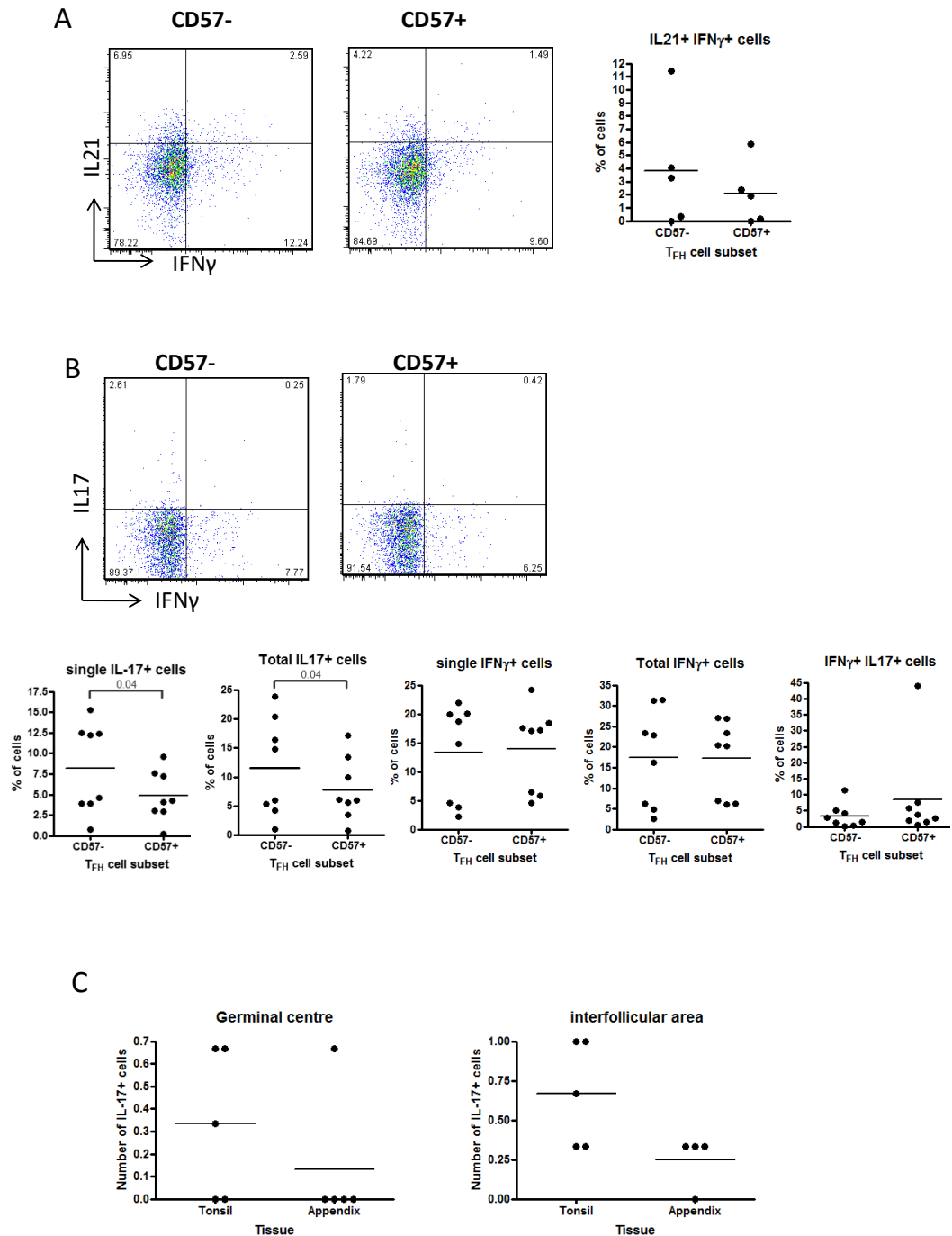


Figure 3-6 Proinflammatory cytokines expressed by T_{FH} subsets

A-B Tonsil mononuclear cells stimulated with PMA and ionomycin for 3 hours A-Left, an example of the flow cytometry separation of IL21+ and IFN γ + cells within the CD57+ and CD57- T_{FH} gates. Right, the percentage of CD57+ and CD57- T_{FH} positive for IL-21 and IFN γ in n=5 tonsil. B- An example of the flow cytometry separation of IL17+ and IFN γ + cells within the CD57+ and CD57- T_{FH} gates and the percentage of CD57+ and CD57- T_{FH} single positive for IL-17 or IFN γ , total IL-17+ or IFN γ + and IL-17+ IFN γ + cells in n=8 tonsil. C- Average number of IL-17 + cells in 1,2 or 3 GCs and

average number of IL-17+ cells in 3 10x10mm of interfollicular area at x40 magnification. Mann-Whitney statistical test performed on A-C.

3.2.3.2 T_{FH} subsets and expression of CD40L

CD40L is a co-stimulatory molecule expressed on the surface of T_{FH} and is required for B cell maturation and survival within the GC. To assess any differences in CD40L expression between T_{FH} subsets, the surface expression of CD40L at basal levels and in response to stimulation was analysed by flow cytometry. Tonsil mononuclear cells were stimulated with PMA and ionomycin for 15 min, 30 min, 1h, 2h and 3h and CD40L expression was assessed at each time point by flow cytometry.

At basal levels there was no significant difference in the surface expression CD40L between CD57+ and CD57- T_{FH} (Fig 3.7B). The trend in expression of CD40L after stimulation over these time points was similar in CD57-, and CD57+ T_{FH} and CD4+ T cells (Fig 3.7A). There was no significant difference in the percentage of CD40L+ cells between CD57- T_{FH} , CD57+ T_{FH} and total CD4+ T cells after 3 hours of stimulation (Fig 3.7C). Also the basal unstimulated level of CD40L did not change after 3 hours in all T cell subsets (Fig 3.7A). Therefore no difference in the expression of CD40L was seen between T_{FH} subsets.

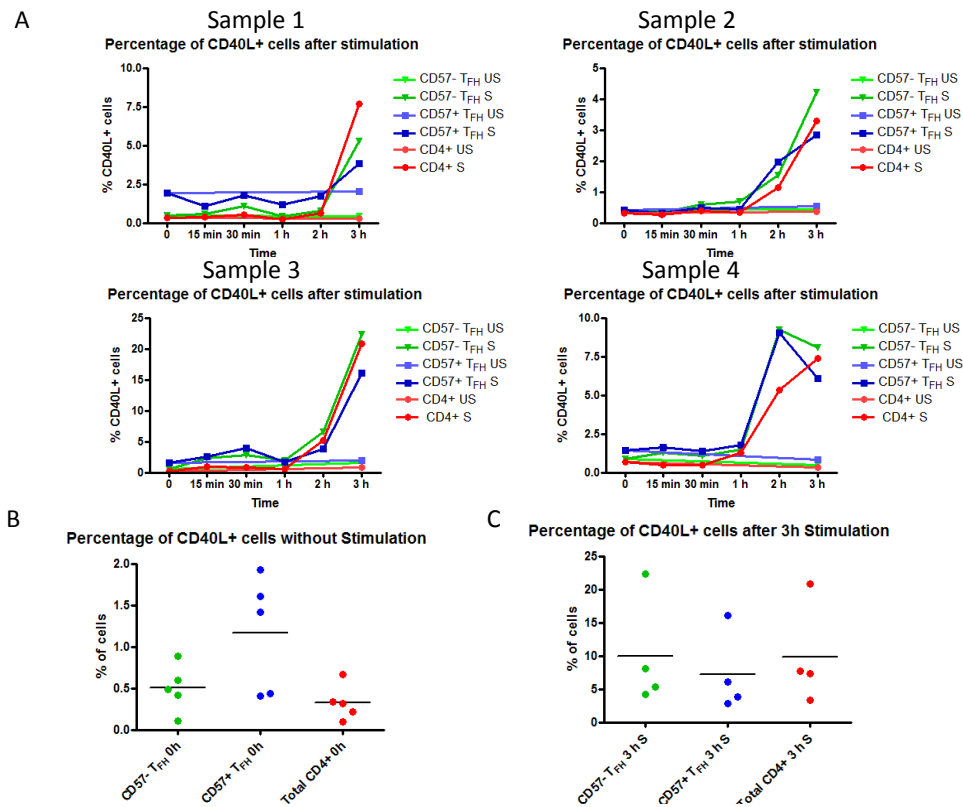


Figure 3-7 CD40L expression on tonsil T cell subsets

A-Flow cytometry analysis of CD40L expression at different time points either unstimulated or stimulated with PMA and ionomycin on 4 different tonsil samples B- CD40L expression on unstimulated tonsil (n=5) T cell subsets. C- CD40L expression on tonsil (n=4) T cells after 3 hours of stimulation with PMA and ionomycin. Mann Whitney statistical test performed on B and C.

3.2.4 Analysis of CD57 expression on T_{FH} subsets over time

As CD57 expression is associated with clonal exhaustion and terminal differentiation, it is possible that CD57- T_{FH} are at an earlier stage of differentiation than CD57+ T_{FH} and CD57- T_{FH} could possibly be precursors for the CD57+ T_{FH}.

Therefore to explore whether CD57- T_{FH} differentiate into CD57+ T_{FH} or vice versa, both populations of tonsil T_{FH} were sorted by FACS (Fig 3.8A). When cell numbers permitted, purity was shown to be 98%. The sorted cells were then labelled with the cell tracking molecule CFSE and cultured for 4 days either alone or with allogeneic tonsil

mononuclear cells isolated at the same time as the sorted T_{FH} (Fig 3.8A). On day four the CD57 expression on the CFSE labelled cells was analysed by flow cytometry.

No difference in expression of CD57 was observed between CFSE labelled T_{FH} cultured alone or with allogeneic tonsil cells (Fig 3.8B & C). After four days of culturing approximately 25% of CD57- T_{FH} expressed CD57 (Fig 3.8B). On the other hand, approximately 10% of CD57+ lost the expression of CD57 (Fig 3.8C).

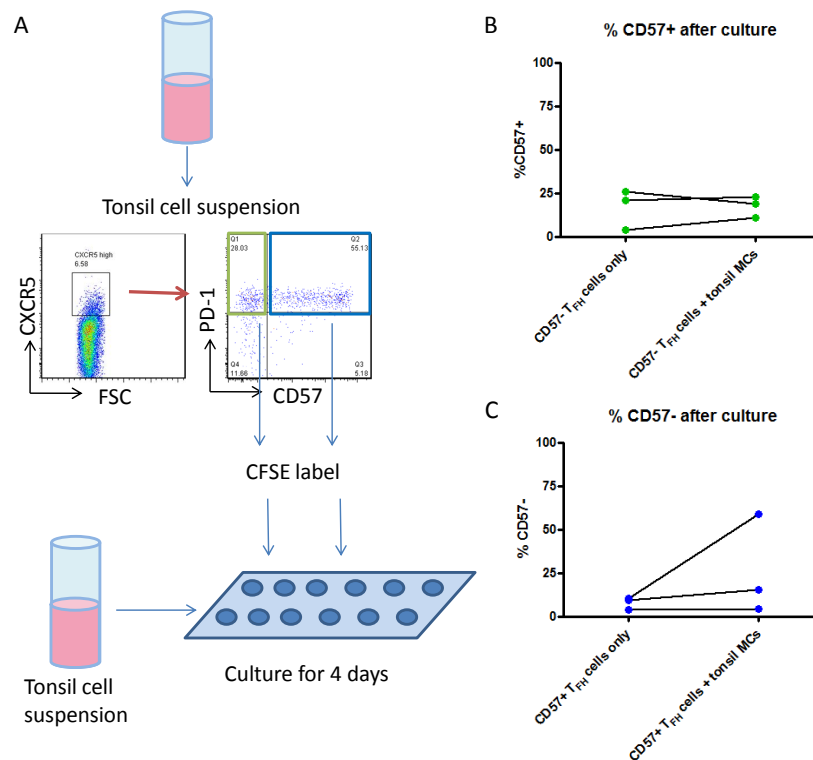


Figure 3-8 Analysis of CD57 expression on sorted T_{FH} subsets

A- Diagram showing the steps involved in isolating and culturing CFSE labelled T_{FH} cells from tonsils. B-The percentage of CD57+ T_{FH} that lost expressed CD57 after 4days of culturing either alone or with tonsil mononuclear cells (n=3). C- The percentage of CD57- T_{FH} that expressed CD57 after 4days of culturing either alone or with tonsil mononuclear cells (n=3). Mann Whitney statistical test performed on B and C.

3.2.5 Analysis of factors known to effect T_{FH} cell frequency

In order to understand the higher frequency of T_{FH} within GALT compared to peripheral lymphoid tissue, factors which have already been shown to effect T_{FH} numbers were explored.

3.2.5.1 Expression of PD-1 on T_{FH} in GALT and Peripheral lymphoid tissue.

PD-1 is a regulatory molecule that controls the proliferation and cytokine production of T_{FH}. PD-1 expression on lymphoid tissue cell suspensions was analysed by FACS. Tonsil tissue was used as an example of peripheral lymphoid tissue and appendix as an example of GALT due to their high lymphoid tissue content.

Analysis of PD-1 on CXCR5⁻, CXCR5⁺ and CXCR5^{high} tonsil and appendix cell suspensions showed a high correlation of PD-1 with CXCR5 on CD4⁺ T cells (Fig 3.9A and B). CXCR5 is highly expressed on T_{FH} and is a defining feature. However, analysis of the CXCR5^{high} CD4⁺ T population, showed no significant difference in the frequency of PD-1^{high} cells between tonsil and appendix tissues (Fig 3.9C).

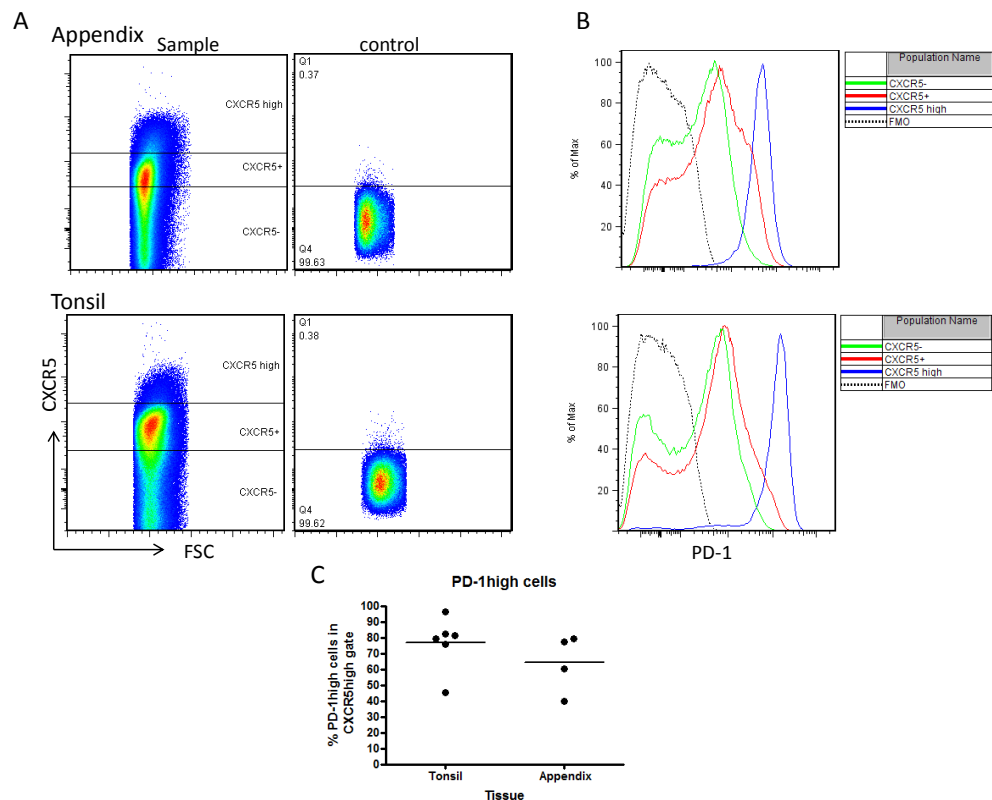


Figure 3-9 Expression of CXCR5 and PD-1 on CD4 T cells in tonsil and appendix cell suspensions.

Whole tonsil and appendix mononuclear cell suspension analysed by flow cytometry. A- CXCR5 expression on CD3⁺ CD4⁺ T cells. CXCR5^{high} cells are defined as the cells expressing levels of CXCR5 higher than the main body of cells and these cells are shaded in blue on these FACS plots. CXCR5⁺ are defined as those cells with higher fluorescence than the control and CXCR5⁻ are those cells with fluorescence under the level of the control B- Histogram of PD-1 expression on CXCR5^{high}, CXCR5⁺ and CXCR5⁻ populations (gates shown on left). C Percentage of PD-1⁺ cells in the CXCR5^{high} fraction of CD4⁺ T cells in tonsil (n=6) and appendix (n=4). Mann Whitney statistical test performed on C.

3.2.5.2 Frequency of Follicular T regulatory cells in lymphoid tissues

Another explanation for the higher frequency in T cells in GALT GCs could be a difference in Tfr frequency. Tfr are a distinct population of T cells that reside in GCs and have regulatory properties. Tfr are related to natural Tregs but play a distinct role in GC regulation (Linterman et al., 2011). A reduction in Tfr is suggested to lead to a reduction in the regulation of T_{FH} and a higher frequency of T_{FH} (Linterman et al., 2011). Therefore the frequency of Tfr was explored between tonsil and appendix.

Analysis of whole tonsil and appendix cell suspension by FACS revealed a higher frequency of Tregs in appendix compared to tonsil (Fig 3.10A). In order to assess Treg frequency within different tissue microenvironments, analysis of foxp3⁺ Tregs on tonsil and appendix tissues was studied by IHC.

The percentage of foxp3⁺ T cells within the interfollicular area of appendix was significantly greater than in tonsil, which is consistent with the data obtained by FACS (Fig 3.10A & C). There was also a trend towards a higher percentage of foxp3⁺ T cells within appendix GCs compared to tonsil, though the difference was not significant (Fig 3.10B). Overall this data shows a higher frequency of Tregs within appendix tissue compared to tonsil and a trend towards a higher frequency of Tfr.

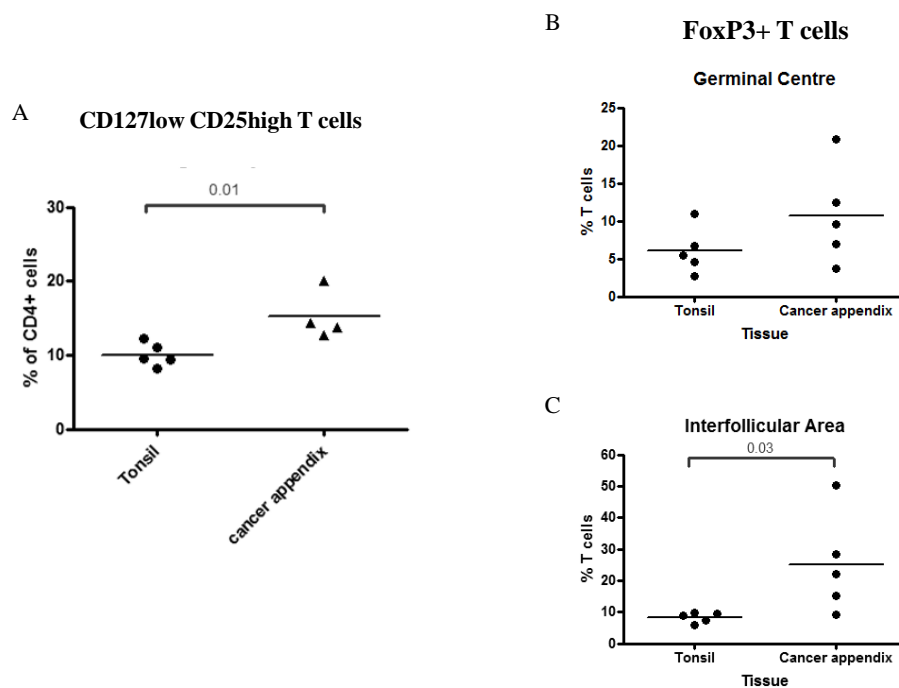


Figure 3-10 Percentage of Treg cells in tonsil and appendix tissue.

A-Percentage of CD127^{low}CD25^{high} T cells in tonsil (n=5) and appendix (n=4) cell suspension assessed by flow cytometry. B Average percentage of foxp3 + T cells within 1, 2 or 3 GCs from tonsil (n=5) and appendix (n=5) C- Average percentage of foxp3 + T cells within 3 10x10mm lymphocyte areas at x40 magnification from tonsil (n=5) and appendix (n=5). Mann Whitney statistical test performed on A-C.

3.3 Discussion

3.3.1 A higher relative density of T_{FH} in GALT compared to peripheral lymphoid tissue

A relatively high frequency of plasma cells secreting polyspecific and autoreactive immunoglobulins generated through a germinal centre response has been observed in the gut mucosa (Benckert et al., 2011). Differences in T_{FH} between GALT and periphery lymphoid tissue have been observed in this study. As T_{FH} play an important

part in the selection and generation of plasma cells it is possible that the differences in T_{FH} may contribute to the differences in specificity of plasma cells found at these sites.

In this chapter, the relative density of T_{FH} within different lymphoid tissues was studied. A higher relative density of T_{FH} was observed in GALT GCs compared to peripheral lymphoid tissue GCs. This has not previously been reported in the literature.

It is thought that T_{FH} frequencies are kept under a tight control to create a selective pressure which selects high affinity B cells to leave the GC to become plasma and memory B cells (Good-Jacobson et al., 2010, Linterman et al., 2011). The higher relative density of T_{FH} in GALT could lower the selective pressure on GC B cells, allowing the generation of plasma cells with a lower affinity for the antigen (Linterman et al., 2011). GC B cells with a lower affinity for the antigen have the possibility of being poly-specific or autoreactive which could explain the observation of autoreactive and poly-specific gut plasmablasts by Benckert et al. (2011).

3.3.2 T_{FH} subsets differ in frequencies between GALT and peripheral lymphoid tissue.

In this investigation, two subsets of T_{FH} have been identified that can be distinguished from one another by their expression of CD57. The proportions of CD57+ and CD57- T_{FH} differ between GALT and peripheral lymphoid tissue. There is a higher relative density of CD57- T_{FH} in GALT but the relative density of CD57+ T_{FH} is consistent in all lymphoid tissues. Therefore the higher frequency of T_{FH} in GALT compared to peripheral lymphoid tissues and is due to a higher frequency of CD57- T_{FH} .

Kim et al. (2005) demonstrated that CD57+ T_{FH} were more efficient at inducing production of IgM, IgG, IgA and IgE by GC B cells than CD57- T_{FH} (Kim et al., 2005, Kim et al., 2001). However, Rasheed et al. (2006) claimed that CXCR5^{high} ICOS+ T_{FH}

had the greatest B cell helper ability regardless to CD57 expression in terms of inducing IgG production .

In this study the relative density of CD57+ T_{FH} was the same in peripheral lymphoid tissue and GALT. Therefore even if CD57- T_{FH} are less able to induce antibody production than CD57+ T_{FH}, they still contributed to a higher relative T_{FH} density that could lower the selective pressure in the GALT GC microenvironment.

3.3.3 *Detecting T_{FH} subsets in cell suspension.*

Some discrepancy can be seen between data sets generated by analysing the percentage of CD57+ T_{FH} by immunohistochemistry or by flow cytometry in this study. The average percentage of CD57+ T_{FH} detected in tonsil by IHC is 90%, whereas the average percentage of CD57+ T_{FH} detected by FC is 57%.

The percentage of CD57+ T_{FH} was lower when tissues were analysed by flow cytometry compared to analysis by immunohistochemistry which would suggest that non-T_{FH} are also contained within the CD57- T_{FH} gate. This is possibly due to FACS techniques being more sensitive and therefore cells with different levels of surface antigen expression are detected. Alternatively this may be a consequence of the definition of T_{FH} in cell suspensions. Unfortunately, a distinguishing marker of T_{FH} has not yet been identified but the CXCR5^{high}PD-1^{high} population has been demonstrated to be a highly effective inducer of antibody production (Rasheed et al., 2006, Wang et al., 2011a). However activated T cells also display similar surface markers as T_{FH} such as CXCR5 and PD-1 (Ansel et al., 1999). Though flow cytometry may not be a precise way to analyse the frequency of T_{FH} in lymphoid tissues, the advantage is the ability to isolate, observe and manipulate live cells which is not possible by immunohistochemistry.

3.3.4 *Differences in cytokine production between T_{FH} subsets*

Difference in the cytokine profile of CD57+ and CD57- T_{FH} was explored in this study. Previous studies demonstrated that T_{FH} can secrete a number of different cytokines but classically T_{FH} are known to secrete IL-21 and IL-4 (Yu and Vinuesa, 2010). In this chapter it was demonstrated that both CD57+ and CD57- T_{FH} were able to produce IL-21 and IL-4 and a small proportion of T_{FH} dual expressed IL-21 and IL-4. However, a significantly higher percentage of CD57- T_{FH} expressed IL-21 compared to CD57+ T_{FH}.

IL-21 has been implicated in the generation and survival of GC B cells and it has been proposed that the effect of IL-21 on GC B cells is an indirect consequence of the role of IL-21 in T_{FH} development (Nurieva et al., 2008). However a recent study has demonstrated that IL-21 can also have a direct effect on GC B cell survival by promoting or maintaining Bcl-6 expression needed for effective affinity maturation (Linterman et al., 2010). Therefore in GALT, the higher frequency of CD57- T_{FH} which produce more IL-21, could promote the survival of B cells with lower specificities which are potentially auto or poly-reactive, through direct effects on Bcl-6 expression and this could contribute to the altered immunoglobulin profile in GALT.

In this chapter, analysis by FC demonstrated that a small percentage of both CD57- and CD57+ T_{FH} produce IL-17. IL-17+ cells were also observed in GCs by IHC, which is consistent with the notion that a proportion of T_{FH} express IL-17. Also a small frequency of IFN γ producing cells was detected in both T_{FH} subsets and along with dual IL-17 and IFN γ producing cells.

There is some debate in the literature as to whether or not T_{FH} produce IL-17 and IFN- γ . Nurieva et al. (2008) states that in mice, T_{FH} do not produce IL-17. However, Bauquet et al. (2009) reported IL-17 expression by T_{FH} in mice immunised with

MOG(35–55) emulsified in CFA. Therefore the antigen type may influence the cytokines that T_{FH} secrete. IFN- γ is expressed by T_{FH} in murine models of viral infections and T_{FH} in IL-21 reporter mice express IFN γ in response to non-specific stimulation (Luthje et al., 2012, Johnston et al., 2009). Bcl-6, is suggested to repress the production of IL-17 and IFN- γ but the effect in T_{FH} seems to be that these cytokines are expressed at lower levels but can then be expressed at higher levels in response to certain antigens (Nurieva et al., 2009).

A higher proportion of IL-17+ cells were detected within the CD57- T_{FH} population compared to CD57+ T_{FH} . Elevated levels of IL-17 have been associated with autoimmune disease (Chen et al., 2010, Wong et al., 2000). Depletion of IL-17 or IL-17 deficient mice has preventative effects or lessens autoimmune disease severity (Nakae et al., 2003, Hofstetter et al., 2005). In particular, down regulation of circulating IL-17+ with a T_{FH} phenotype has been associated with the amelioration of lupus like symptoms in mice (Wu et al., 2008). As a higher proportion of CD57- T_{FH} produce IL-17, they may promote the generation of autoreactive B cells more than CD57+ T_{FH} . Since CD57- T_{FH} are found at a higher density in GALT, the IL-17 they produce may contribute to the autoreactive B cells found in the gut.

3.3.5 ***CD40L expression by T_{FH} subsets***

Further analysis of the CD57- and CD57+ T_{FH} subsets in tonsil demonstrated that they both upregulate CD40L upon stimulation. CD40L is stored intracellularly and upon activation, CD40L is shuttled to the cell surface where it engages with CD40 on B cells to provided survival signals for the maturation of GC B cells (Koguchi et al., 2012, Elgueta et al., 2009). In this study there was no significant difference in the frequency of CD40L+ CD57- and CD57+ T_{FH} after 3 hours of stimulation therefore suggesting that

both T_{FH} subsets can equally provide the same level of B cell help through CD40-CD40L interactions.

Another point to consider is that in this chapter T_{FH} subsets from tonsils were used to study cytokine production and CD40L expression. However, it is possible that in GALT the T_{FH} cytokine profile may be different. Due to difficulties in obtaining sufficient number of lymphocytes from gut tissue, it was not possible to analyse cytokine production of T_{FH} cells in GALT. However sufficient cells numbers were collected from one healthy appendix sample and data indicates that T_{FH} cells subsets from appendix show a similar pattern of cytokine production as those in the tonsil (data not shown).

3.3.6 *CD57- T_{FH} could be precursors of CD57+ T_{FH}*

In this study, the expression of CD57 on T_{FH} subsets was analysed after four days of culturing. On day four, a higher percentage of T_{FH} gained CD57 expression than lost expression of CD57 suggesting that T_{FH} are more likely to progress from CD57- to CD57+ rather than the other way round. Previous publications suggest that T_{FH} cells develop when T cells from the T cell zone which are cognate for invading antigen are activated and migrate to the T cell-B cell border. Interactions with B cells activate signalling pathways that allow the T cell to enter the GC as a pre-T_{FH} (Baumjohann et al., 2011, Kerfoot et al., 2011). It is possible that newly formed pre-T_{FH} are the CD57- T_{FH} observed in GCs and as T_{FH} develop further they start to express CD57 (Kim et al., 2001).

The gut environment is constantly being challenged by antigens from the gut lumen and T_{FH} are constantly being induced. The increase in frequency of CD57- T_{FH} in GALT compared to peripheral lymphoid tissues could be due to a higher turnover of T_{FH}.

However, it is also possible that the difference in the percentage of cells converting from CD57⁺ to CD57⁻ and vice versa, is related to differences in the rate of cell death between CD57⁻ and CD57⁺ T_{FH}. As CD57⁺ T_{FH} may be clonally exhausted, their rate of death may be greater than CD57⁻ T_{FH}. Therefore, it would appear that a smaller proportion of cells were progressing from CD57⁺ to CD57⁻.

3.3.7 ***PD-1 and T_{FH} frequency***

PD-1 expression on T_{FH} was explored in this study because engagement of PD-1 on the surface of T_{FH} with one of its receptors, PD-L1 or PD-L2, controls T_{FH} responses and proliferation (Hams et al., 2011). However there is conflicting opinions to the effect of PD-1 deficient on GC B cells. Good-Jacobson et al. (2010) describes a population of higher affinity GC B cells arising from PD-1 signalling deficiency but this was due to a high death rate of GC B cells and only high affinity B cells remained. Depletion of PD-1 has also been linked with the development of autoimmunity (Nishimura et al., 1999, Nishimura et al., 2001). Autoimmunity in PD-1 deficient mice may in part be due to the expansion of T_{FH} and altered T_{FH} cytokine profile which leads to a dysregulated antibody response (Hams et al., 2011, Kawamoto et al., 2012).

Theoretically, a lower expression of PD-1 on T_{FH} would relieve the regulatory effects of PD-1 and could lead to an increase in the T_{FH} population and less stringent selection of GC B cells. However the expression of PD-1 was comparable on T_{FH} in GALT and peripheral lymphoid tissue, therefore a lower expression of PD-1 does not account for the higher frequency of T_{FH} cells observed in GALT.

A recent study by Kawamoto et al. (2012), highlights the importance of PD-1 in the regulation of IgA specificity in the gut. In the gut of *pdcd1*^{-/-} mice, IgA had an altered specificity to gut bacteria and therefore an altered bacteria colonisation ensued. An expansion of T_{FH} was observed in *pdcd1*^{-/-} GALT and GC B cells underwent less

stringent clonal selection (Kawamoto et al., 2012). Overall it seems that PD-1 can alter the dynamics of germinal centre responses by the frequency of cells that express it in mice in this study and potential also in humans based on the data in this thesis.

3.3.8 *Role of follicular T regulatory cells in the frequency of T_{FH}*

Linterman et al. (2011) recently described a population of Tregs which reside in GCs and appear to control T_{FH} numbers and are termed follicular T regulatory cells. As there is a higher frequency of T_{FH} in GALT compared to peripheral lymphoid tissue, this might suggest that GALT has a lower frequency of Tfr. Surprisingly, the data in figure 3.11 suggests that there is a higher frequency of Tregs in the gut and a trend towards a higher percentage of Tfr in appendix compared to tonsil.

However, in GALT, the higher frequency of Tregs and Tfr could be a result of the higher frequency of T_{FH} . It is possible that GCs with a higher number of T_{FH} contain a higher number of Tfr to keep the number of T_{FH} at a constant frequency. Therefore the higher frequency of T_{FH} seen in GALT is not due to a dysregulation of Tfr but regulatory T cells in the gut are also at a higher frequency to correlated with the higher frequency of T_{FH} and therefore set both of these T cell populations at equilibrium.

3.4 *Conclusions*

Overall, in this chapter several differences between GALT and peripheral lymphoid tissue T_{FH} have been observed that may contribute to the differences in the properties of plasma cells induced at these sites. In conclusion:

- A higher relative density of T_{FH} was observed in GALT compared to peripheral lymphoid tissues.
- Two subsets of T_{FH} have been identified by their expression of CD57. The proportions of these two subsets differ between lymphoid tissues.

- The higher relative density of T_{FH} in GALT is due to a higher frequency of CD57- T_{FH} .
- CD57- T_{FH} are possibly newly formed or precursors for CD57+ T_{FH} and their higher frequency in GALT could reflect a higher turnover of T_{FH} .
- No difference in the expression of PD-1 was found between lymphoid tissues.

4 Chapter 4: Investigation of Clonally Related Cells Within and Between T Follicular Helper Cells and Regulatory T Cells Subsets

4.1 Introduction

At the time that this project was started the literature on T_{FH} was beginning to gather pace and issues of lineage and plasticity were largely unknown. However recently, several studies have investigated the origin and development of T_{FH} and the field has moved on. Analysis of T_{FH} by affymetrix microarrays revealed that they have a distinct transcriptional profile which differs from Th1 and Th2 cells (Chtanova et al., 2004). It has been demonstrated in mice that T_{FH} cells can develop from antigen specific native CD4 T cells that are activated within the lymphoid tissue and independently of other T cell subsets (Baumjohann et al., 2011, Nurieva et al., 2008). Epigenetic studies suggest that T_{FH} cells can also arise from other Th subsets as active marks have been found on *tbx21*, *rorc* and *gata3* loci in T_{FH}-like cells (Lu et al., 2011). These genes encode the master regulators for Th1, Th17 and Th2 lineages respectively. Studies using an IL-4 reporter mice have demonstrated that IL-4 producing T_{FH} cells within the germinal centre also express GATA3 during a helminth infection (Zaretsky et al., 2009). As GATA3 is the master transcriptional factor that controls Th2 lineage, this may indicate that these T_{FH} are derived from a Th2 lineage (Zaretsky et al., 2009).

In 2009, Tsuji et al demonstrated that foxp3⁺ Tregs transferred into CD3 ϵ ^{-/-} mice were able to down-regulate foxp3 and reside within the GCs of PP. They gained T_{FH} like features such as the expression of Bcl-6, CD40L, PD-1 and CXCR5 and were able to aid B cell expansion and plasma cell formation (Tsuji et al., 2009). This suggested a possible developmental link between Tregs and T_{FH} cells.

Tregs promote tolerance and repress immune responses whereas T_{FH} have a “helper” phenotype and promote a mature humoral immune response. These two lineages could be seen as having opposing roles in the immune system. Tsuji et al. (2009) suggests that in the gut the same signals are needed for the generation of T_{FH} and Tregs, such as antigen engagement through the TCR. It is then the environment that the cell is in that influences whether its fate is to become a Treg or T_{FH}. This relationship may be important in the gut to tip the balance of Treg and T_{FH} in favour of a humoral response or immune suppression.

However, the majority of studies into T cell plasticity have used murine models and there is very little evidence that T_{FH} are developmentally related to Tregs in humans.

To look at the developmental relationship between cell subsets in humans requires a different approach to that used in murine models. As it is not possible to track a cells fate *in vivo* by, for example, adoptive transfer or use of reporter genes in humans, evidence of developmental origins can only be studied retrospectively or *in vitro*. During T cell development, the TCR gene segments are rearranged creating near unique DNA sequences. The TCR sequence is retained by any progeny of that cell. Therefore clonally related T cells can be identified by analysis of the TCR DNA sequence. By using this method, common ancestry or the developmental relationship between T_{FH} and Tregs can be explored.

4.1.2 Aims:

To identify and isolate Treg populations from lymphoid tissue by flow cytometry for further analysis.

To investigate any clonal relationship between T_{FH} and Tregs and within these T cell populations from peripheral blood and tonsil by sequence analysis of rearranged TCR γ sequences amplified by clone specific primers.

To investigate any clonal relationship between T_{FH} and Tregs and within these T cell populations from tonsil by sequence analysis of rearranged TCR β sequences from a restricted TCR β repertoire.

4.2 Results

The initial stage of this study involved optimization of staining and gating for identification of Tregs and their subsets in tonsil tissues which will be described below. Parameters for identification of T_{FH} were optimised in chapter 3.

4.2.1 Isolating regulatory T cells from lymphoid tissue

In order to study the potential for a clonal relationship between T_{FH} cells and Tregs, purified populations of cells were isolated from lymphoid tissue by flow cytometry. Most studies of human Tregs that use flow cytometry to identify Tregs, use blood. It was therefore important to ensure that an equivalent population was identified and isolated from tonsils.

As in blood, CD4⁺, CD14⁻, CD127^{low}, CD25^{high} cells were present in tonsil (Fig 4.1A). However, further analysis of this T cell subset by inclusion of PD-1 and CD45RA in the panel of antibodies identified heterogeneity that has not been described before and this was analysed further (Fig 4.1B).

Foxp3, a transcription factor that is commonly used to identify Tregs, was used to determine which T cell subsets found in the CD127^{low}CD25^{high} gate included Tregs (Fontenot et al., 2003). The PD-1^{high} cells were mainly Foxp3⁻ and therefore not classed as Tregs (Fig 4.1C). CD45RA⁺ cells expressed intermediate levels of foxp3 and the

CD45RA-PD-1^{low} and CD45RA-PD-1⁻ cells expressed higher levels of foxp3 (Fig 4.1C). The latter two subsets are consistent with the phenotypes of Treg cells described by others (Miyara et al., 2009).

A high expression of PD-1 is associated with T_{FH} (Fig 4.2A). Therefore to further explore the identity of the CD127^{low}CD25^{high} PD-1^{high} T cells in tonsils, the expression of CXCR5 on these cells was analysed. CD127^{low}CD25^{high} PD-1^{high} cells in tonsils also had a high expression of CXCR5 (Fig 4.2B). In blood, there are very few PD-1⁺ T cells within the CD127^{low}CD25^{high} gate and the PD-1^{high}CXCR5^{high} population identified in tonsil was not observed in blood (Fig 4.2 A and B). As both PD-1 and CXCR5 are expressed by T_{FH} this suggests that T_{FH} localise within the gates used for isolation of Treg when tonsil tissue rather than blood is analysed. Therefore, to isolate Tregs from tonsil without contamination by T_{FH}, a new gating strategy that gated out the PD-1⁺ T cells within the CD127^{low}CD25^{high} gate was used (Fig 4.3A).

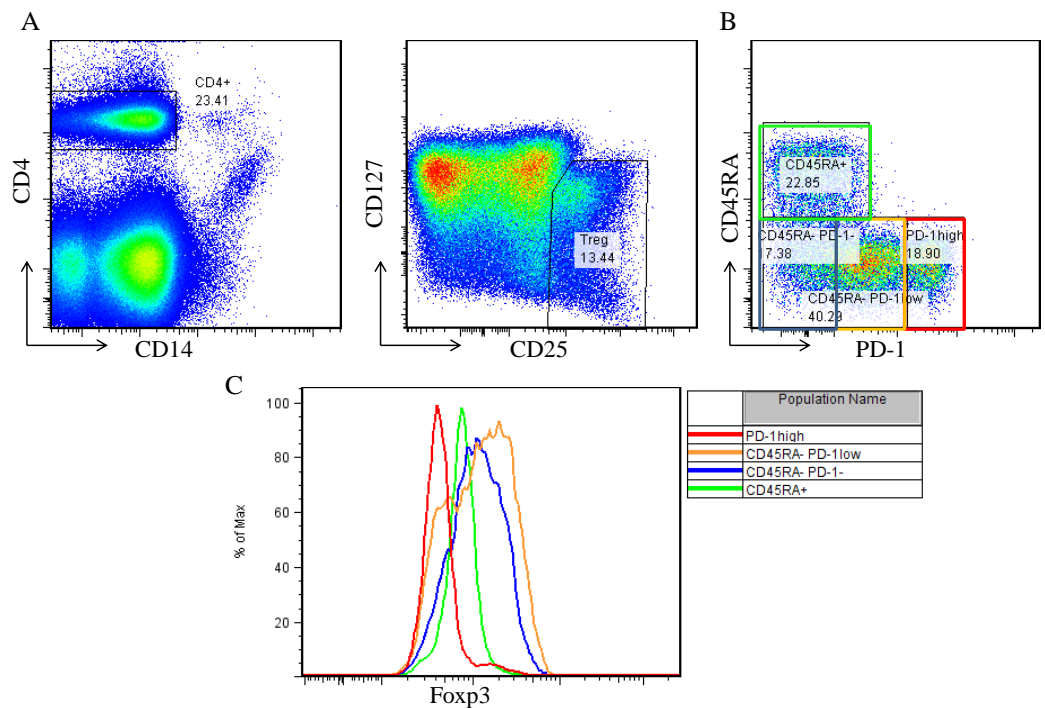


Figure 4-1 Foxp3 expression in cell subsets within the CD127^{low}CD25^{high} T cell population.

One example tonsil lymphocytes analyzed by flow cytometry to identify Treg cells (n=3). A- An example of gating on CD4⁺ CD14⁻ T cells (left) Gating example for CD127^{low}CD25^{high} within the CD4 T cell population (right) B- Populations of T cells within the CD127^{low}CD25^{high} gate separated by expression of CD45RA and PD-1. C- Expression of foxp3 in the populations shown in B.

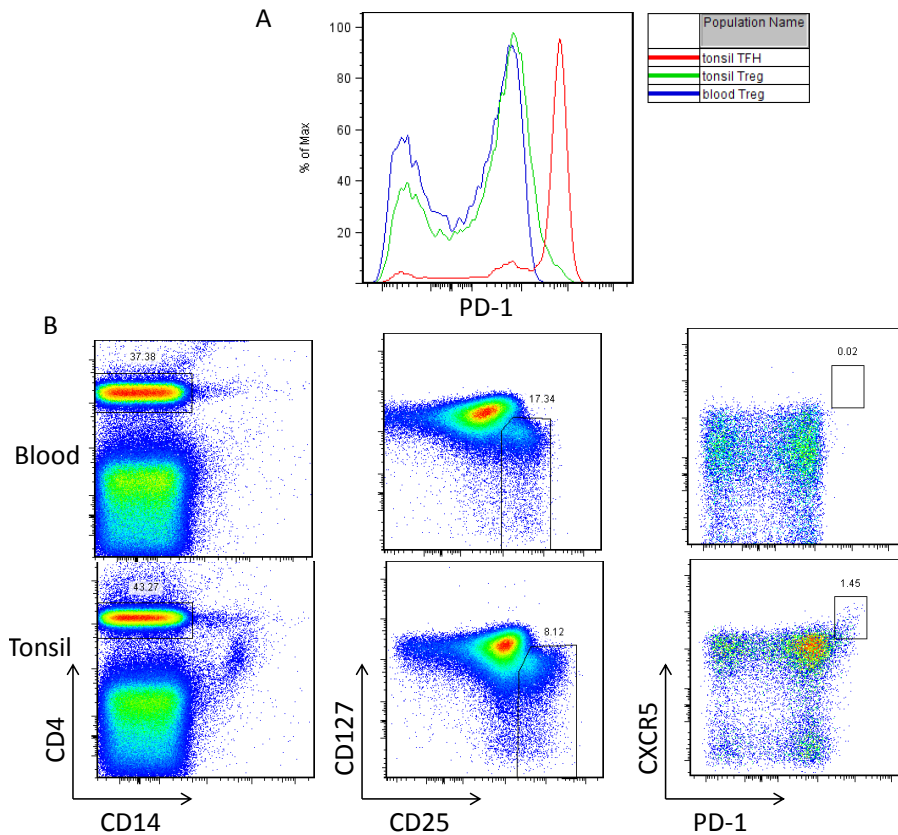


Figure 4-2 Expression of T_{FH} cell markers within the CD127^{low}CD25^{high} T cell population.

Flow cytometry analysis of T cell subsets in paired blood and tonsil from one healthy individual. A- Histogram of PD-1 expression in tonsil CXCR5^{high} T_{FH} cells, tonsil CD127^{low}CD25^{high} Tregs and blood CD127^{low}CD25^{high} Tregs. B- Gating for CD4⁺ CD14⁻ T cells (far left) gating for CD127^{low}CD25^{high} within the CD4 T cell population(middle), and gating for CXCR5^{high}PD-1^{high} with in the CD127^{low}CD25^{high} T cell population. Lymphocytes from blood (top) and tonsil cell suspension (bellow).

4.2.2 *Frequency of Treg subsets in lymphoid tissue and peripheral blood.*

Tregs can be divided into three subsets based on their expression of CD45RA and CD25. Naïve Tregs are CD45RA⁺, CD25^{intermediate}, memory Tregs are CD45RA⁻,

CD25^{intermediate} and activated T regs are CD45RA⁻, CD25^{high}. The frequency of activated Treg subsets was similar in tonsil and peripheral blood. However there was a higher frequency of memory Tregs within tonsils and a lower frequency of naive T regs (Fig 4.3B).

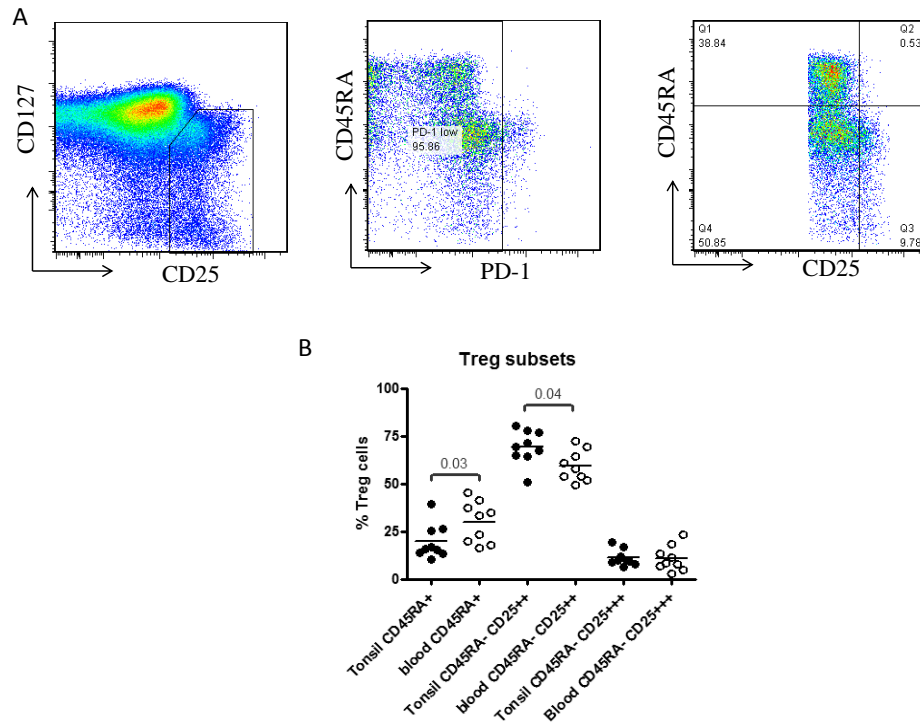


Figure 4-3 Frequencies of Treg subsets in tonsil and blood.

A-An example of tonsil lymphocytes previously gated on CD4 T cells. Gating for CD127^{low}CD25^{high} T cells (far left), gating for PD-1^{low} cells within the CD127^{low} CD25^{high} subsets (middle) and separation of PD-1^{low} CD127^{low}CD25^{high} T reg by CD45RA and CD25 (far right). B- Percentage of Treg subsets generated by the gating strategy in A in unpaired tonsil and blood. Mann-Whitney statistical test performed on B.

4.2.3 *Searching for clonality between T cell populations by comparing germline TCR- γ chain sequences.*

The first method used to investigate the possibility that a clone of T cells could span different T cell subsets, was sequence analysis of unused rearrangements of the TCR γ

chain. Clones of T cells within each subset and that span these two T cell subsets were sought by comparison of the junctional sequences generated at the TCR gamma locus. The method devised by Golby et al. (1999) initially used to identify and analyse groups of sequences from populations of T cells. Subsequently identical TCR sequences were sought by designing primers specific to the junctional region of the TCR γ chain.

4.2.3.1 Direct analysis of diversity and comparison of sequences.

Fresh peripheral blood from a healthy donor was sorted for T reg cells and circulating T_{FH} cells (cT_{FH}) and tested for evidence of clonality between circulating populations of T_{FH} and Treg. T regs were sorted by the phenotype CD4+CD25+CD127^{low} (Fig 4.4A) and cT_{FH} cell by CD3+CD4+CD57+CXCR5+ (Fig 4.4B). The DNA was extracted from both of these subsets and 11 PCRs using the V γ 11, V γ 101 and J γ 11 primers described by McCarthy et al. (1992) were performed for each T cell subset. The PCR products were cloned and 5-7 clones from each PCR were sequenced.

Sequence analysis of 102 TCR γ sequences from both cT_{FH} and Tregs showed that both of these populations of T cells were extremely diverse and there were no repeated sequences even between sequences from the same PCR. No related sequences between cT_{FH} and Tregs were identified. Analysis of the number of nucleotides used in the junctional region of these clones revealed that Treg TCR γ sequences had significantly longer junctional regions compared to cT_{FH} (Fig 4.4C & D).

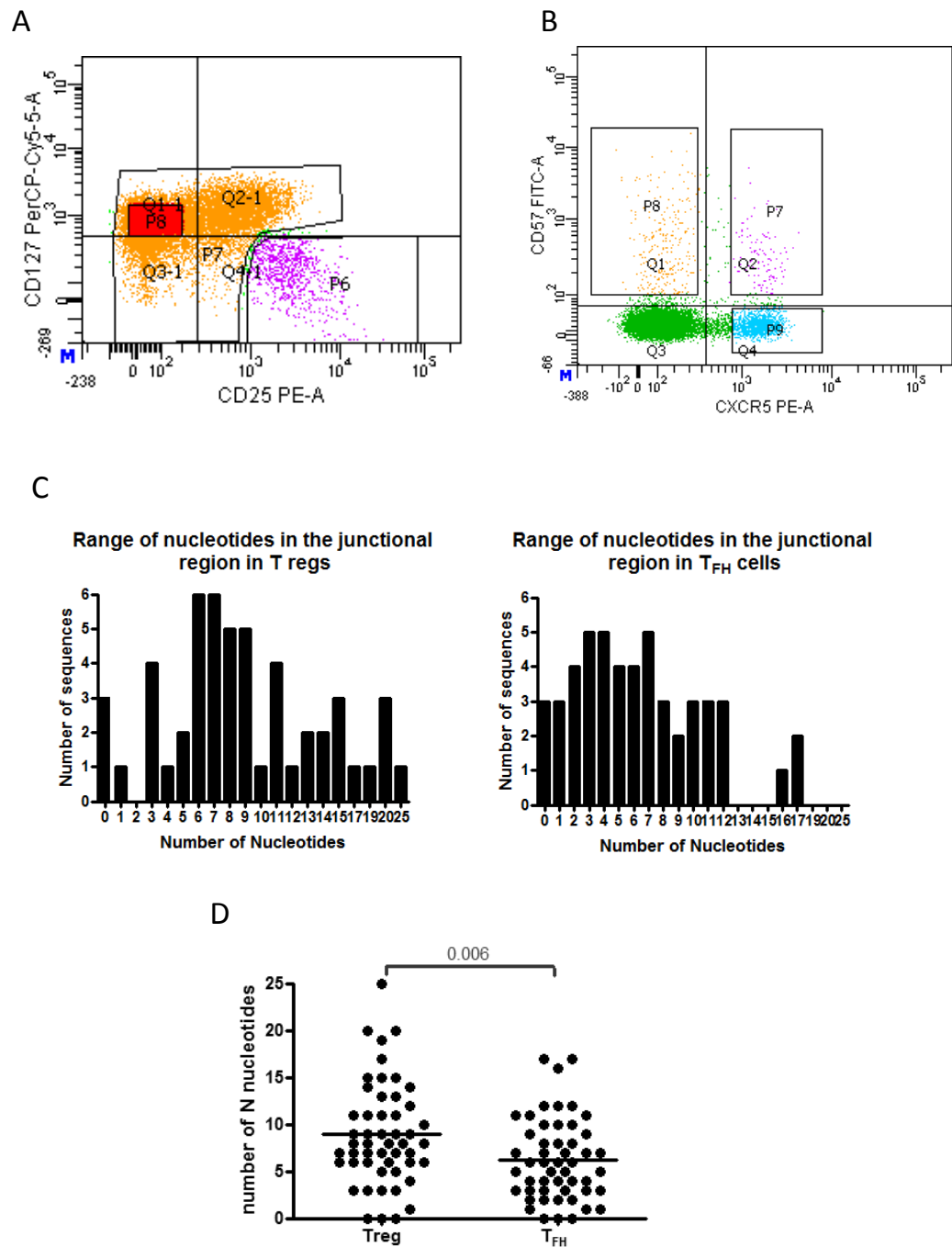


Figure 4-4 Sequence analysis of sorted Treg and cT_{FH}

A and B- FACS plots of cell sort on fresh peripheral lymphocytes from a healthy individual. A- CD4⁺ cells sorted for CD127^{low}CD25^{high} T reg cells (P6). B- CD3⁺CD4⁺ cells sorted for CXCR5⁺CD57⁺ T_{FH}. C- Number of nucleotides in junctional region in T reg and cT_{FH}. D- Comparison of the number of nucleotides in the junctional region between T reg and T_{FH}. Unpaired T statistical test performed on D.

4.2.3.2 Searching for related cells with clone specific PCR primers

To amplify clonally related T cells, clone specific primers were designed to sequences with N regions greater than 12bp and originating from both cT_{FH} and Treg populations. The clone specific primers were complimentary to the junctional region but 2 or more nucleotides at the end of junctional region were not included in the primer. The end nucleotides of the junctional region not encoded by the primer were used to confirm colonicity between sequences (Fig 4.5).

		10	20	30	40	50	60	70	80
		V segment				junctional region		J segment	
A07 seq	TTCTGGGGTCTATTACTGTGCCACCTGGGACGGG	CTCCCCAACGTGT <u>GG</u>							
A07 primer						TTATAAGAAACTCTTTGGCAGTGGAAACAACACTTG			
A08 seq	TTCTGGGGTCTATTACTGTGCCACCTGGGACGGG	CCGCGAACGCCAGC <u>GC</u>							
A08 primer						ATTATAAGAAACTCTTTGGCAGTGGAAACAACACTTG			
D10 seq	TTCTGGGGTCTATTACTGTGCCACCTGGGAT	GGGCCTGGGTAG <u>AG</u>							
D10 primer						TATTATAAGAAACTCTTTGGCAGTGGANCAACACTTG			
E11 seq	TTCTGGGGTCTATTACTGTGCCACCTGGGACAGG	CATCTCCCCACACGCTATGGGGT <u>GAGG</u>							
E11 primer						TTATAAGAAACTCTTTGGCAGTGGAAACAACACTTG			
E08 seq	TGATTCTGGGGTCTATTACTGTGCCACCTGGGA	AGTGCTTTTGGGT <u>TG</u>							
E08 primer						AGAAACTCTTTGGCAGTGGAAACAACACTTG			
E03 seq	TTCTGGGGTCTATTACTGTGCCACCTGGGACG	CACGATAATTT <u>AA</u>							
E03 primer						AATTATTATAAGAAACTCTTTGGCAGTGGAAACAACACTTG			
F04 seq	TTCTGGGGTCTATTACTGTGCCACCTGGGATAG	TTAGGAGTTAGTATG <u>CAT</u>							
F04 primer						GAATTATTATAAGAAACTCTTTGGCAGTGGAAACAACACTTG			
F09 seq	TTCTGGGGTCTATTACTGTGCCACCTGGGACGGG	CGAACGTGTTGTGGG <u>GG</u>							
F09 primer						TTATAAGAAACTCTTTGGCAGTGGAAACAACACTTG			
E12 seq	TTCTGGGGTCTATTACTGTGCCACCTGGGATAG	CGAGGAGCCTAC <u>CA</u>							
E12 primer						TATTATAAGAAACTCTTTGGCAGTGGAAACAACACTTG			
F07 seq	TTCTGGGGTCTATTACTGTGCCACCTGGGAT	GGGCTGGCTC <u>GG</u>							
F07 primer						TATTATAAGAAACTCTTTGGCAGTGGAAACAACACTTG			
J14 seq	TCTGGGGTCTATTACTGTGCCACCTGGGACGG	CGCGCCGCGGAAAAACA <u>AAC</u>							
J14 primer						TTATAAGAAACTCTTTGGCAGTGGAAACAACACTTGAATCACTAG			
H11 seq	TTCTGGGGTCTATTACTGTGCCACCTGGGACGGG	CCTGCCGTCACGT <u>TGG</u>							
H11 primer						TTATAAGAAACTCTTTGGCAGTGGAAACAACACTTG			
G05 seq	TTCTGGGGTCTATTACTGTGCCACC	CCCCCCTACCCCT <u>TA</u>							
G05 primer						TTTATAAGAAACTCTTTGGCAGTGGAAACAACACTTG			

Figure 4-5 Clone specific primers to TCR γ junctional regions of sequences from circulating T cell populations.

Original TCR γ sequences separated into the V segment, junctional region and J segment with the clone specific primer below. The 2 or more nucleotides of the junctional region not included in the primer are underlined.

13 primers were designed from sequences originating from cT_{FH} and Treg. PCRs were performed using the clone specific primers and the PCR product which yielded the original sequence. All 13 clone specific primers amplified a sequence of the correct size (positive control) (Fig 4.6A). The clone specific primers were tested with DNA from multiple donor whole lymphocytes as a negative control. 4 clone specific primers amplified products of the correct size and were excluded for possibly being non-specific (Fig 4.6B). The remaining 9 clone specific primers were used in PCRs with the DNA from all the cT_{FH} and Treg PCR products generated using V γ 11, V γ 101 and J γ 11 primers (Fig 4.6C).

The only primer designed from a T_{FH} cell TCR γ sequence, F09, did not pick up any other products of the same size in any of the PCRs (table 4.1). Primer E08 yielded bands of the correct size in 5 other Treg PCR products and A07 gave a band of the correct size in one other Treg PCR product but neither of these primers produced a band with any of the cT_{FH} PCR products. Primers E12, A08 and D10 yielded products of the correct size with both T_{FH} and Treg PCR products (table 4.1). These PCR products of the correct size amplified by E12, A08 and D10 clone specific primers from DNA originating from cT_{FH} were cloned and two examples from each PCR were sequenced.

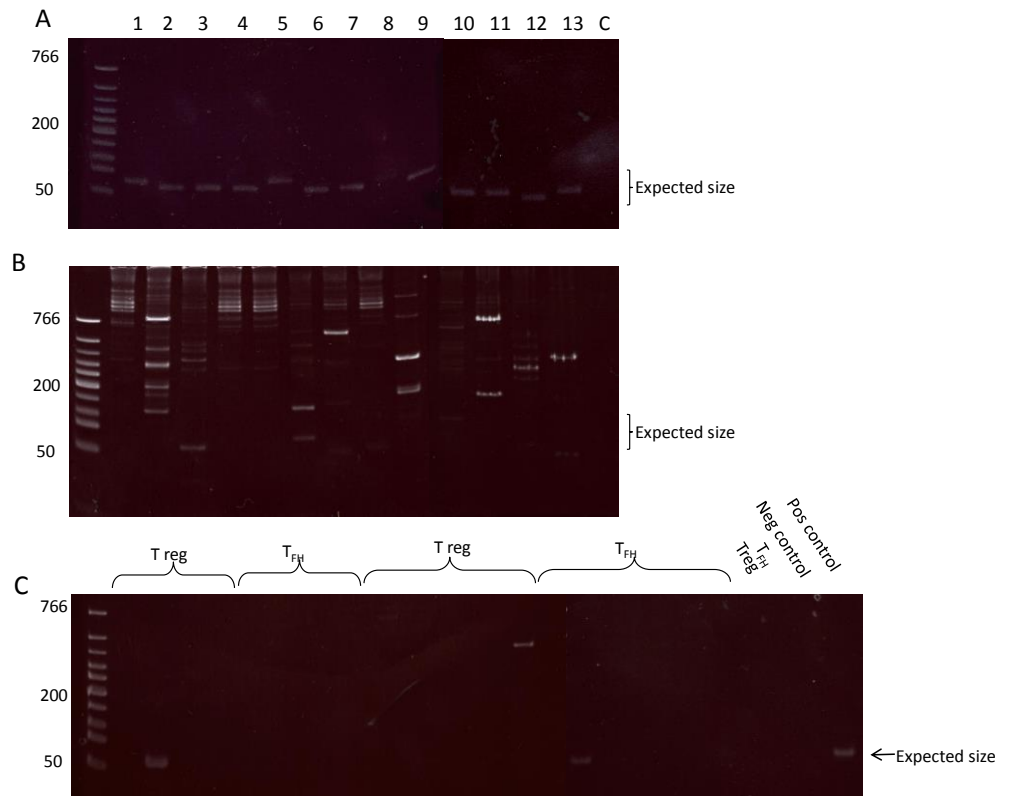


Figure 4-6 Screening for related clones in Treg and T_{FH} cell subsets using clone specific primers

PCR products run on 10% polyacrylamide gel. A- Positive control for the 13 clone specific primers where the primers were used to amplify the DNA from the original PCR reaction that the clone was sequenced from. B- Negative control for the primers using a multi donor buffy coat DNA amplified for the J γ chain. C- An example of the DNA amplified by clone specific primer T reg E12.

Primer	T _{FH} target	T reg target
T _{FH} F09	0/10	0/11
Treg E08	0/11	5/10
Treg A07	0/11	1/10
Treg E12	1/11	1/10
Treg A08	1/11	1/10
Treg D10	1/11	1/10
Treg E11	0/11	0/10
Treg F04	0/11	0/10
Treg J14	0/11	0/10

Table 4-1 Number of PCR reactions which gave a band of the correct size for each primer.

The ratios represent how many PCR products yielded a band of the correct size when the clone specific primer was applied out of all the other PCR products for that T cell subset. The PCR product from which the original sequence came from is not represented.

The cT_{FH} sequences, amplified by the Treg clone specific primers A08, D10 and E12, were not identical to the original Treg sequences. Even though the PCR product and cloned sequences were the correct size the sequences were different (Fig4.7).

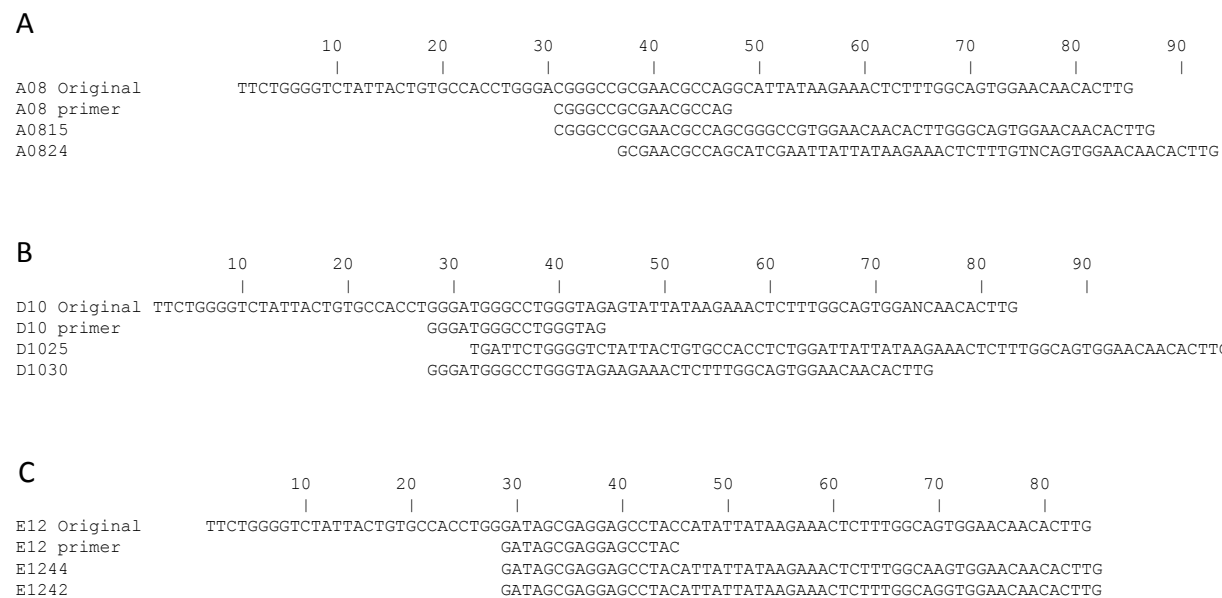


Figure 4-7 Alignment of sequences amplified by TCR γ clones specific primers

A-A0815 and A0824 from a T_{FH} PCR compared to the original A08 sequence and primer. B D1025 and D1030 from a T_{FH} PCR compared to the original D10 sequence and primer. C- E1244 and E1242 from a T_{FH} PCR compared to the original E12 sequence and primer.

Thus, no evidence of clonal relatedness between Treg and cT_{FH} from blood was observed.

4.2.4 Searching for clonality between T cell populations from peripheral lymphoid tissue

No clonality was found in circulating populations of T cell populations and this could be due to vast diversity and number of T cells found within peripheral blood and therefore clonally related cells may be found more readily in tissue. Therefore, the methods above were applied to T cell subsets from lymphoid tissue.

4.2.4.1 Direct analysis of diversity and comparison of sequences

Several T_{FH} and Treg subsets were sorted from two tonsils (TON-12 and TON-14) by flow cytometry to explore clonality not only between T_{FH} and T reg but also within T_{FH} subsets and Treg subsets. T_{FH} cells were defined as CD3+CD4+CXCR5^{high}PD-1+ as described in chapter 3 and T_{FH} subsets were divided into CD57+ and CD57- subsets. However, CD57+ and CD57- CXCR5^{high}PD-1- T cells (non-T_{FH}) were also sorted to explore if there is a clonal relationship between these subsets and T_{FH} (Fig 4.8A). Treg were defined as CD3+CD14-CD127^{low}CD25^{high}PD-1^{low} as described in figure 4.3A and divided into CD45RA+ and CD45RA- subsets. CD127^{low}CD25^{high}PD-1+ CD4 T cells (PD-1+ non-Treg) were also sorted. CD127^{low}CD25^{low} CD4 T cells were sorted at the same time as the Treg subsets, as a control population (control T cells) (Fig 4.8A and table 2).

Phenotype	Population name
CD3+CD4+CXCR5 ^{high} PD-1+CD57+	CD57+ T _{FH}
CD3+CD4+CXCR5 ^{high} PD-1+CD57-	CD57- T _{FH}
CD3+CD4+CXCR5 ^{high} PD-1-CD57+	CD57+ non-T _{FH}
CD3+CD4+CXCR5 ^{high} PD-1-CD57-	CD57- non-T _{FH}
CD3+CD14-CD127 ^{low} CD25 ^{high} PD-1 ^{low} CD45RA+	CD45RA+ Treg
CD3+CD14-CD127 ^{low} CD25 ^{high} PD-1 ^{low} CD45RA-	CD45RA- Treg
CD3+CD14-CD127 ^{low} CD25 ^{high} PD-1+	PD-1+ non Treg
CD3+CD14-CD127 ^{low} CD25 ^{low}	Control T cells

Table 4-2 Phenotype and population name of sorted T cell subsets.

2 or 3 TCR γ chain PCRs were performed for all subsets of T cells described in table 4.2. Rather than sequencing a number of PCR products from all the T cell subsets which would be costly and time consuming, only PCR products of CD57+ T_{FH} and PD-1+non-Treg subsets were sequenced in the first instance. In figure 4.1 PD-1 non-Tregs were dismissed as being Tregs as they did not express foxp3, their presence in the CD127^{low}CD25^{high} gate could indicate that they are related to Tregs. CXCR5 was also expressed on a proportion of PD-1 non-Tregs which could indicate that they are T_{FH}. Sequence analysis of PD-1 non-Tregs may give an insight into whether these cells are related to Tregs, T_{FH} or both.

From TON-12, 23 sequences were analysed from the CD57+ T_{FH} cell subset and 24 sequences were analysed from the PD-1+non-Treg subset. From TON-14, 23 sequences were analysed in the CD57+ T_{FH} cell subset and 22 sequences were analysed from the PD-1+non-Treg subset. Analysis of these sequences found that the T cell populations were extremely diverse as all the sequences were different to each other and no clonally related sequences were observed. No significant difference in the number of nucleotides

at the junctional region was found between PD-1+ non Tregs and CD57+ T_{FH} (Fig 4.8C). This might indicate that PD-1+ non-Tregs are more similar to T_{FH} than Tregs as circulating Tregs tended to have longer junctional regions.

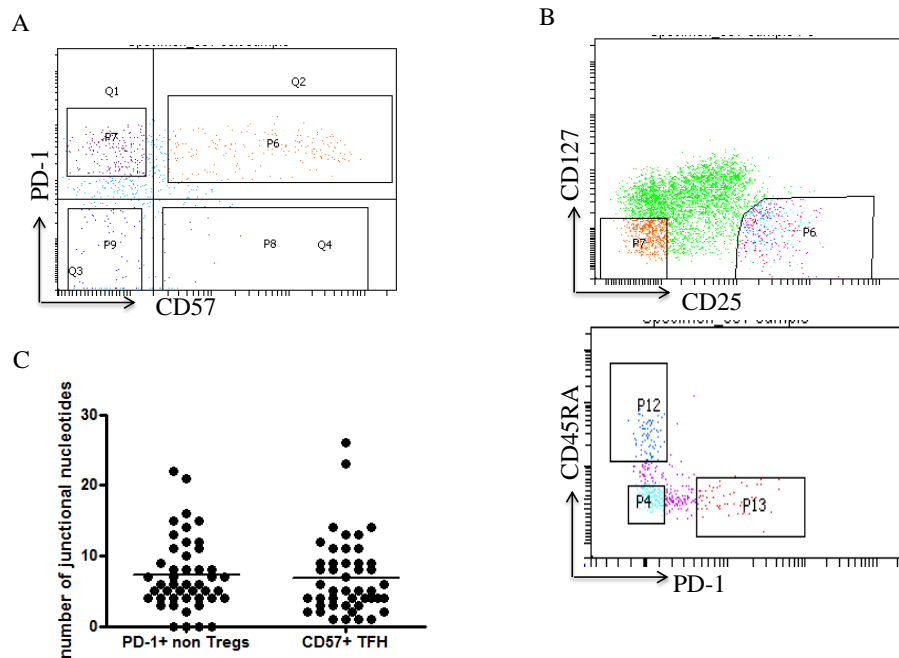


Figure 4-8 Generation of clone specific primers from TCR γ chain sequences.

A and B- Examples of gating for sorting T cell populations by flow cytometry. A- An example of the FACS gating for subsets of CXCR5^{high} CD4 T cells. Cells in gates P6, P7, P8, P9 were sorted. B- An example of the gating for control T cells and CD127^{low}CD25^{high} T cells (top). An example of gating for the subsets within the CD127^{low}CD25^{high} gate (bottom). Cells in gates P4, P7, P12 and P13 were sorted. C- number of nucleotides in the junctional region of the TCR γ in PD-1+ non Tregs and CD57+ T_{FH}. Mann-Whitney statistical test performed on C.

4.2.4.2 Searching for related cells with clone specific PCR primers

From the TCR γ sequences, clone specific primers were designed to junctional regions longer than 12 bp. For TON-12, primers were designed to 3 sequences from the CD57+ T_{FH} and 4 sequences from the PD-1+non-Tregs. From TON-14, primers were designed

to 2 sequences from CD57⁺ T_{FH} and 4 sequences from the PD-1⁺non-Treg (table 4.2). In the cases where the junctional regions were relatively short, two primers were designed. One primer of 16 nucleotides in length, indicated in red, and the other 18 nucleotides in length, which also includes the nucleotides highlighted in green (table 4.3).

PCR	Sequence	V segment	N segment	J segment
TON 12 CD57+ TFH PCR 1	A1	TTCTGGGGTCTATTACTGTGCCACCTGGGACAGG	CTTTGCCCCCTACGGTATATGGG	CTCTTTGGCAGTGAACAACACTTG
TON 12 CD57+ TFH PCR 2	B2	TGATTCTGGGGTCTATTACTGTGCCACCTGGGACAGG	CCATAACTATAAAGACGCTAACATCC	GAAACTCTTTGGCAGTGAACAACACTTG
TON 12 CD57+ TFH PCR 3	B6	TTCTGGGGTCTATTACTGTGCCACCTGGGAT	GGGCGGAGAGGG	ATTATAAGAAACTCTTTGGCAGTGAACAACACTTG
TON 12 PD-1+ non-Treg PCR 1	D1	TTCTGGGGTCTATTACTGTGCCACCTGGGA	GCTTCTACGTACCGC	TAAGAAACTCTTTGGCAGTGAACAACACTTG
TON 12 PD-1+ non-Treg PCR 2	D3	TGATTCTGGGGTCTATTACTGTGCCACCTGGGAC	GGGCGAAGGGTATTGC	ATTATTATAAGAAACTCTTTGGCAGTGAACAACACTTG
TON 12 PD-1+ non-Treg PCR 3	D12	TTCTGGGGTCTATTACTGTGCCACCTGGGACA	AGAATCAACCCAGCCGACGG	AAGAAACTCTTTGGCAGTGAACAACACTTG
TON 14 CD57+ TFH PCR 1	E1	TCTGGGGTCTATTACTGTGCCACCTGGGAT	GGTCCCCGTAGTGT	GAAACTCTTTGGCAGTGAACAACACTTG
TON 14 CD57+ TFH PCR 2	E9	TTCTGGGGTCTATTACTGTGCCACCTGGGATAG	CCCCTACGGACCTT	TTATTATAAGAAACTCTTTGGCAGTGAACAACACTTG
TON 14 PD-1+ non-Treg PCR 1	G2	TTCTGGGGTCTATTACTGTGCCACCTG	ACCCCGAAAAACGTC	TTATAAGAAACTCTTTGGCAGTGAACAACACTTG
TON 14 PD-1+ non-Treg PCR 1	G7	TGATTCTGGGGTCTATTACTGTGCCACCTGGGAC	TTTACCTTGTGGGAGGTGTTGG	AGAAACTCTTTGGCAGTGAACAACACTTG
TON 14 PD-1+ non-Treg PCR 2	H1	TTCTGGGGTCTATTACTGTGCCACCTGGGAC	CGCACCAACATTTTC	TTATTATAAGAAACTCTTTGGCAGTGAACAACACTTG
TON 14 PD-1+ non-Treg PCR 2	H8	TTCTGGGGTCTATTACTGTGCCACCTGGGATA	AGGAGCCCCTTTG	TTATAAGAAACTCTTTGGCAGTGAACAACACTTG

Table 4-3 TCR γ chain sequences used to generate clone specific primers

Sequences from tonsil 12 and tonsil 14 T cells with N segments containing 12 or more nucleotides. Nucleotides highlighted in red are the primer sequence. Where both a 16 and 18 nucleotide primer was designed, the two extra nucleotides in the 18 nucleotide primers are highlighted in green.

The primers were tested with the PCR product from which the sequence was originally identified as a positive control and all but one primer (D3 18) passed this positive test (Fig 4.9A). To negatively test for non-specific binding of the clone specific primers, DNA from whole lymphocytes from multiple donors was used as a template. The primers that amplified product of the same size as the target sequence were excluded at this stage.

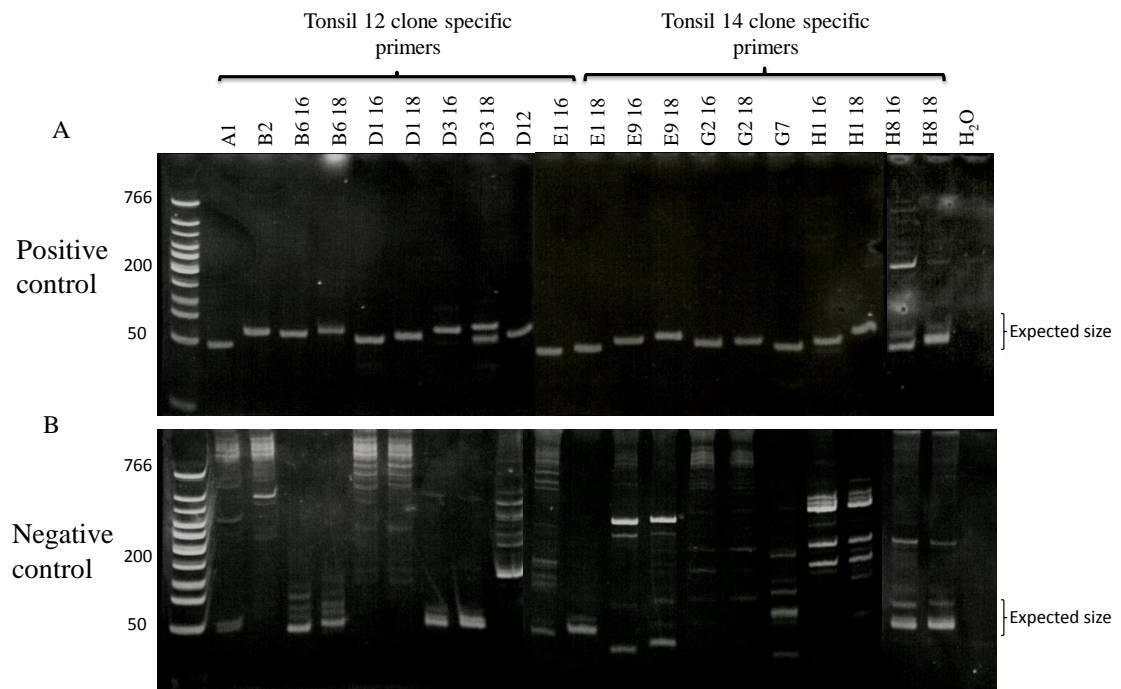


Figure 4-9 Positive and negative test PCRs for clone specific primers

A- Positive control PCR to test clone specific primers designed from sequences in table 2. Purified PCR product from which the sequences originated was used as a DNA template. B- Negative control PCR to test clone specific primers where DNA extracted from multiple donor buffy coats was used.

From TON-12, one clone specific primers from the CD57+ T_{FH} (B2 primer) and 2 clone specific primers (D1 16 and D12 primers) from the PD-1+non-Treg passed this selection process. From TON-14, one clone specific primers (E1 16 primer) from the CD57+ T_{FH} and 2 clone specific primers (G2 18 and H1 16 primers) from the PD-1+non-Treg passed (Fig 4.9B).

The selected clone specific primers were used with DNA from PCR product from all the other T cell subsets originally sorted from the tonsil which included Treg subsets and T_{FH} subsets (Fig 4.8A and table 4.2).

For TON-12, there were no other sequences of the correct size amplified by the CD57+ T_{FH} B2 primer and PD-1+non-Treg D1 16 primer apart from the original positive control (Fig 4.10A and B). The PD-1+non-Treg D12 primer amplified sequences of the correct size in DNA from CD57- T_{FH}, CD57+ T_{FH} cells, another PD-1+non-Treg PCR product and CD45RA- Treg cells (Fig 4.10C).

For TON-14, the CD57+ T_{FH} E1 primer amplified sequences of the correct size from CD57- T_{FH} and CD45RA- Tregs (Fig 4.11A). The PD-1+non-Treg G2 primer amplified sequences of the correct size from CD57- T_{FH} and CD57+ T_{FH} (Fig 4.11B). The CD57+ T_{FH} H1 primer amplified sequences of the correct size from CD45RA- Tregs and the control T cells (Fig 4.11C).

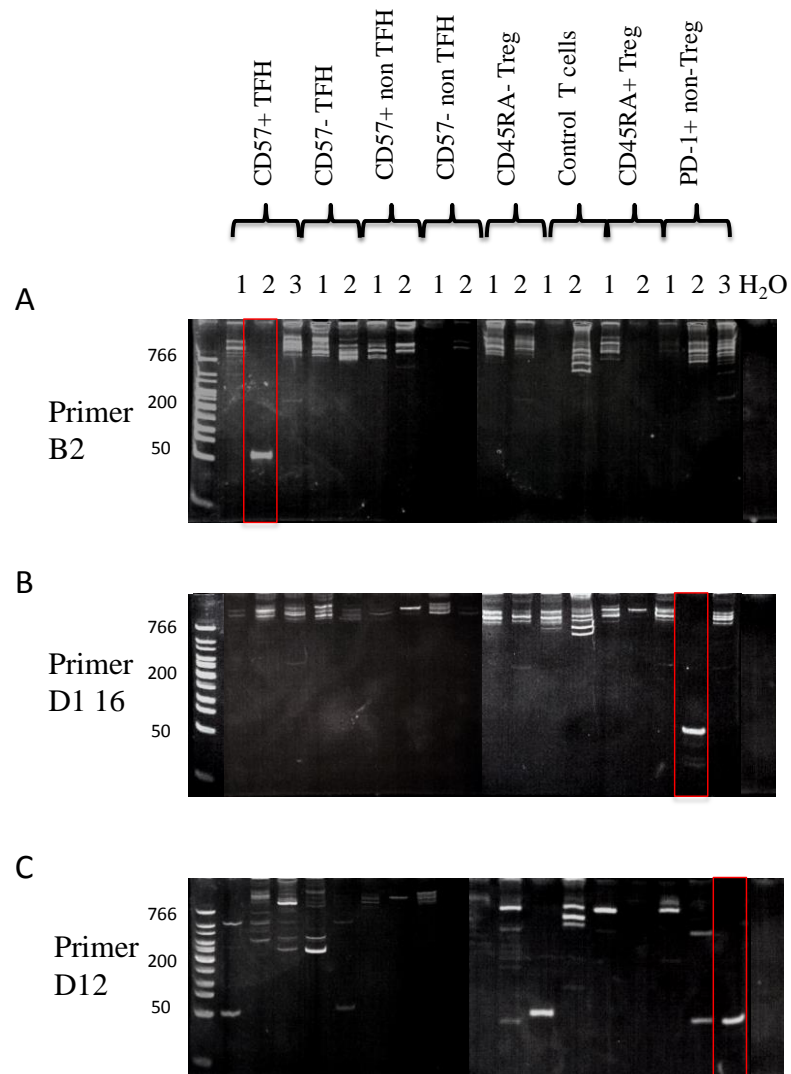


Figure 4-10 TON 12 PCR products amplified by clone specific primers

Purified PCR product from amplified TCR γ sequence from all sorted T cell subsets was used as a template for clone specific primers and TCR γ J primers. The PCR containing DNA from which the clone originated is highlighted in red. A- PCR PCR products using B2 clone specific primer. B- PCR products using D1 16 clone specific primer. C- PCR products using D12 clone specific primer

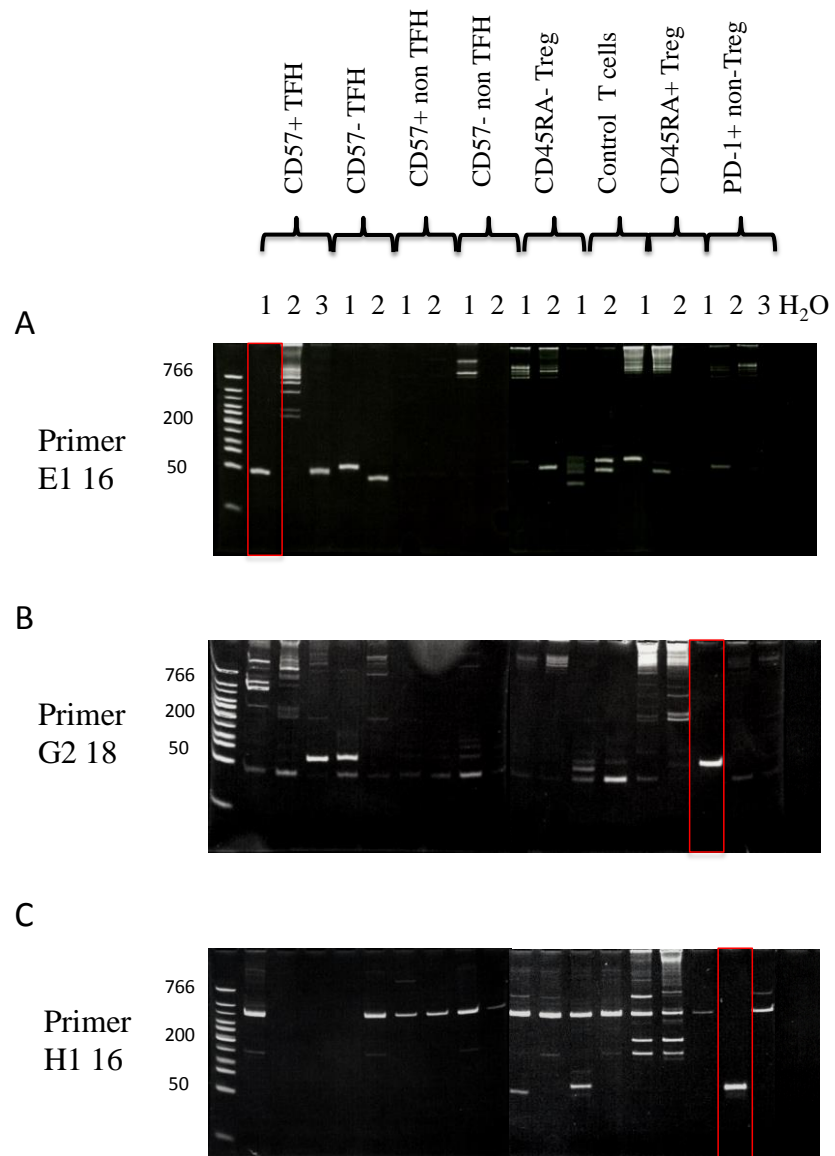


Figure 4-11 TON 14 PCR products amplified by clone specific primers

Purified PCR product from amplified TCR γ sequence from all sorted T cell subsets was used as a template for clone specific primers and TCR γ J primers. The PCR containing DNA from which the clone originated is highlighted in red. A- PCR products using E1 16 clone specific primer. B- PCR products using G2 18 clone specific primer. C- PCR products using H1 16 clone specific primer.

PCR products which yielded a band of the correct size were cloned and sequenced but none of them matched the original sequence that the primer was designed from (Fig 4.12). Therefore despite extensive searching no clonal relationship was found within T cell populations or between T_{FH} and Tregs.

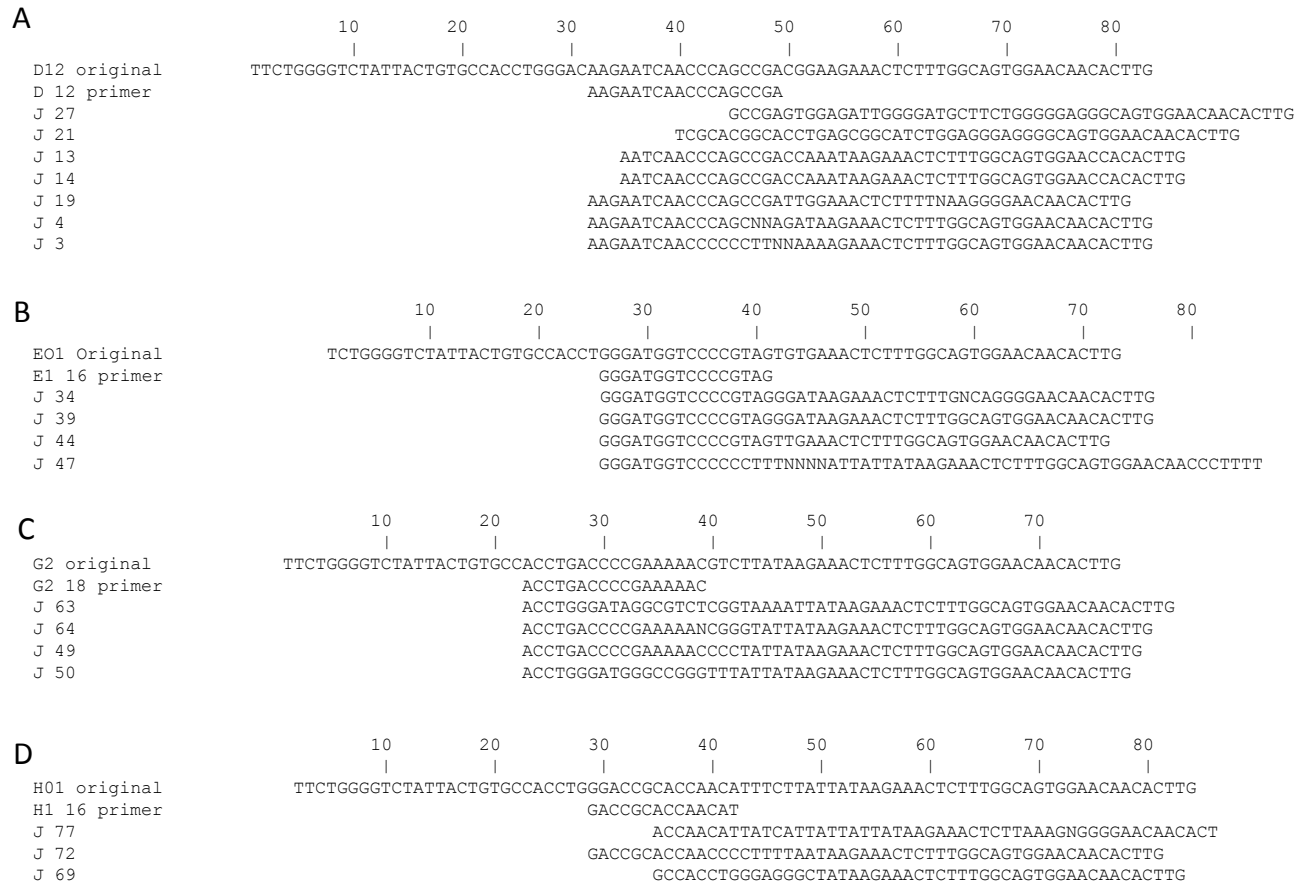


Figure 4-12 Comparative alignment of sequences amplified by clone specific primers

A- J3 and J4 from CD57+ T_{FH} PCR 1, J13 and J14 from CD57- T_{FH} PCR 2, J19 and J21 from control T cells PCR 2 and J27 from PD-1+non-Treg PCR 2 compared to the original D12 sequence and primer.

B- J39 and J44 from CD57- T_{FH} PCR 1 and J47 from CD45RA- Treg PCR 2 compared to the original E02 sequence and primer.

C J49 and J50 from CD57+ T_{FH} PCR 3 and J63 and J64 from CD57-T_{FH} PCR 1 compared to the original G2 sequence and primer. J69 and J72 from CD45RA- Treg PCR 1 and J77 from control T cells PCR 1 compared to the original H01 sequence and primer.

4.2.5 *Searching for clonality between T cell populations by comparing TCR- β chain sequences.*

No evidence of clonality was found between or within any of the T cells subsets studied when analysing the TCR γ by either analysing the sequences or with clone specific primers. This might have been due to the high diversity of sequences which were identified by the TCR γ chain primers. In order to increase the probability of identifying related TCR sequences from different T cell subsets and within T cell populations, a method of detecting only part of the population was applied. A similar concept enabled identification of clonally related B cells in a diverse population by using PCR primers to a fraction of the total immunoglobulin repertoire (Boursier et al., 2005).

Primers to TCR β V03 and J1.5 were designed so only a small fraction of the TCR β repertoire would be amplified. The DNA from TON-14 CD57+T_{FH}, CD57- T_{FH}, CD45RA+ Treg, CD45RA- Treg and control T cells isolated by the method shown in figure 4.8 A and B and table 4.2, was used as the template in the TCR β repertoire PCRs. For each subset of T cells, different DNA was amplified in 5 TCR β PCRs (Fig 4.13). The PCR product was cloned in bacterial plasmids and sequenced. 83 sequences were analysed overall and the number of sequences for each PCR and subset is shown in table 3.

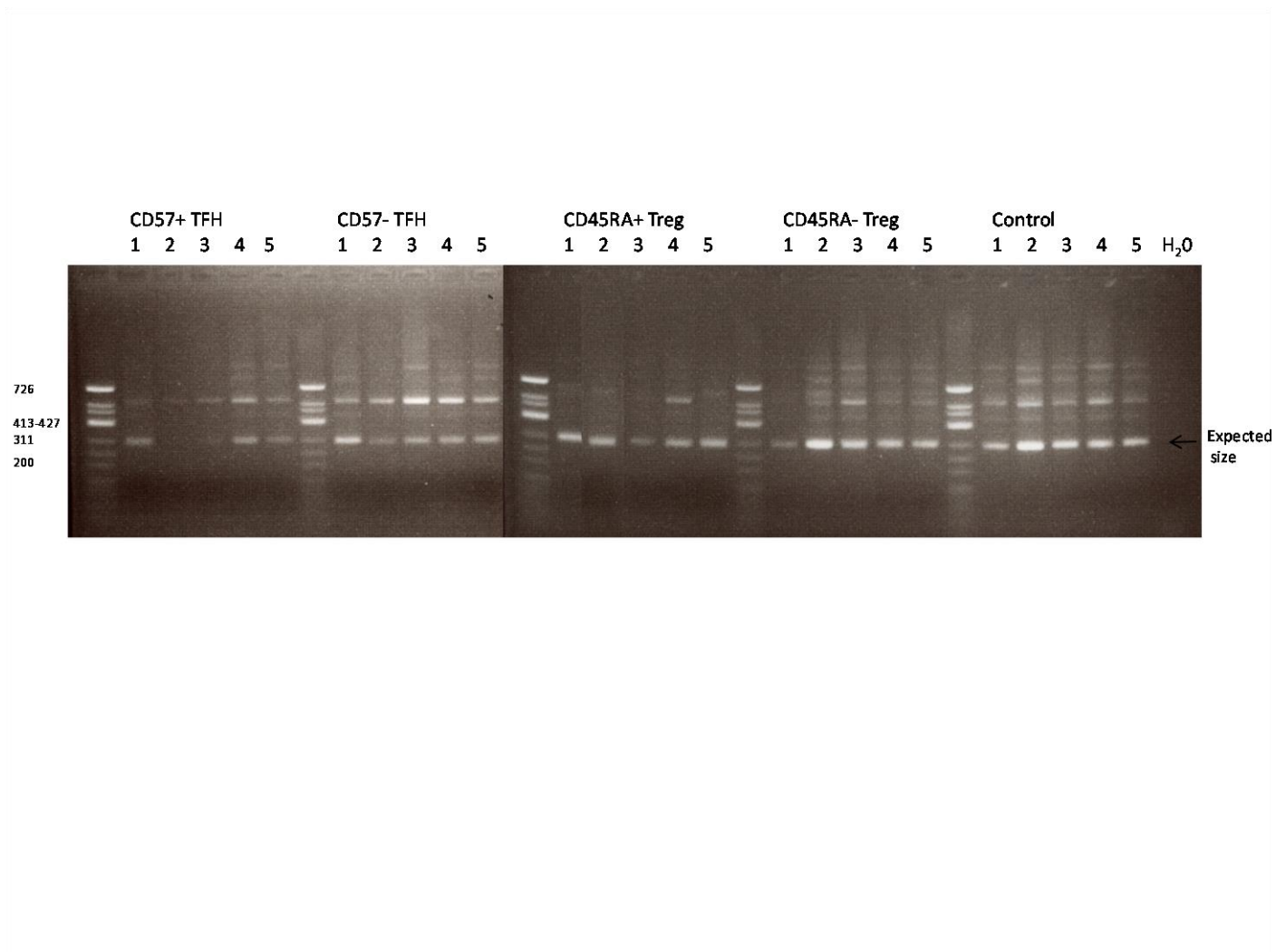


Figure 4-13 TCR β PCR products from Treg and T_{FH} subsets

PCR products from PCRs amplifying V03 and J1.5 TCR β sequences. 5 different PCRs were performed for each T cell subset and the PCR product was run on 1.5% Agarose gel. The correct product size was visualized at approximately 300bp.

T _{FH} CD57+					T _{FH} CD57-					Treg CD45RA+					Treg CD45RA-					Control				
P C R 1	P C R 2	P C R 3	P C R 4	P C R 5	P C R 1	P C R 2	P C R 3	P C R 4	P C R 5	P C R 1	P C R 2	P C R 3	P C R 4	P C R 5	P C R 1	P C R 2	P C R 3	P C R 4	P C R 5	P C R 1	P C R 2	P C R 3	P C R 4	P C R 5
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2	3	2	3	4	4	3	3	3	4	4	5	4	5	3	4	3	2	3	5	4	4	3	3	3

Table 4-4 TCR beta sequences from Tonsil 14 sorted T cell subsets

Each row represents a unique TCR beta sequence and the number of times the sequence appeared in a single PCR is indicated by the number in that cell.

48 unique sequences were identified. A smaller number of unique TCR β sequences were amplified in each PCR using this method due to a high frequency of repeated sequences within PCR products. The repeated sequences in each PCR were a consequence of the PCR only. This meant that performing more PCRs for sequencing is more advantageous than sequencing more colonies from the same PCR to increase the data set of sequences from different cells.

Two examples of the same gene rearrangements in different PCRs were observed. The first example (light grey row) was a gene rearrangement derived of DNA from CD57+ and CD57- T_{FH} as well as from the control T cell population (table 4.3). The second gene rearrangement (dark grey row) was observed in several different PCR products containing DNA from CD57+ T_{FH} and also from control T cells (table 4.3). However, no clonality was observed between tonsil T_{FH} and Treg subsets using this method of sequence analysis of a fraction of the TCR β repertoire.

4.3 Discussion

4.3.1 *Frequencies of Treg subsets in peripheral blood and lymphoid tissues.*

In this chapter, a method of detecting Tregs in lymphoid tissue was established. In lymphoid tissues, a population of PD-1+ cells which are foxp3- was detected in the CD127^{low}CD25^{high} gate used to identify Tregs. Adjustments to the gating strategy excluded this population. Using this gating strategy, comparisons between Treg subsets in tonsil and blood found a higher frequency of memory Tregs and a lower frequency of naive Tregs within tonsil tissue.

Most analysis of the properties of human Tregs uses circulating Treg populations found within peripheral blood. However lymphoid tissue is believed to play an important part in the maturation and induction of Tregs. Naive Tregs produced in the thymus circulate in the blood and enter lymphoid tissue where they can become activated (Lee et al., 2007). Once activated, Tregs gain the ability to home to non-lymphoid tissues (Lim et al., 2006, Lee et al., 2007). As Tregs mature from a naive phenotype to an activated phenotype they lose their expression of CD45RA. Both naive and memory/active Tregs have been previously reported to reside in tonsil tissue alongside non-treg T cells and tonsil CD4⁺CD25⁺ Tregs have been shown to be suppressive (Lim et al., 2006, Taams et al., 2001). Lim et al. (2006) suggests that a higher frequency of naive CD45RA⁺ Tregs (49%) is found in tonsil compared to peripheral blood (17%) as naive Tregs home straight to lymphoid tissue and emerge into the peripheral blood as CD45RA⁻ activated/ memory Tregs. However the finding in this chapter were that there are less naive CD45RA⁻ Tregs in tonsil compared to blood. Discrepancies in the frequency of CD45RA⁺ and CD45RA⁻ Tregs could be due to differences in methods used to detecting Tregs. Lim et al. (2006) detected Tregs by foxp3 and CD4 only whereas in this study CD4, CD127 and CD25 were used together to isolate Tregs. Foxp3 is also transiently upregulated on activated non suppressive T cells and these cells could be included when only foxp3 expression is used to detect Tregs (Wang et al., 2007).

The higher frequency of memory CD45RA⁻ Tregs and lower frequency of CD45RA⁺ Tregs detected in tonsil tissue in this chapter could reflect the tonsil as a site of activation and therefore Tregs are constantly losing the expression of CD45RA. Whereas the blood would contain newly emerging naive Tregs as well as activated Tregs from lymphoid tissues which could lower the frequency of activated Tregs.

4.3.2 *Are T follicular helper cells and regulatory T cells developmentally related?*

In this chapter no clonality was identified between circulating populations of T_{FH} and Tregs. TCR γ and TCR β sequence analysis of tonsil T_{FH} and Treg also found no clonality between these populations.

There could be several reasons as to why no developmental relationship has been observed between T_{FH} and Tregs in this study, but a developmental relationship was identified in the study by Tsuji et al. (2009) For example, this phenomenon could be an idiosyncratic effect only seen in CD3 ϵ ^{-/-} mice.

It is possible that the environment is important in the conversion of Tregs to T_{FH}. In the CD3 ϵ ^{-/-} mice, conversion of foxp3⁺ T cells to T_{FH}-like cells only occurred in PP and not in spleen or lymph nodes (Tsuji et al., 2009). The gut naturally has autoreactive antibodies generated through GC responses. It is possible that the T_{FH} cell progeny from natural Tregs would be reactive to auto antigens. It is possible that the mucosal environment favours the conversion of Tregs from a regulatory phenotype to a helper phenotype. However why this could only occur in the gut and if it occurs in humans at all remains unclear. Unfortunately, due to time constraints and difficulty in obtaining and isolating T_{FH} and Treg subsets from fresh gut tissue, we were unable to study clonality of T_{FH} and Treg from human gut mucosa.

Several studies have analysed the shared TCR repertoire between Treg and Tconv in disease models (Wong et al., 2007, Nguyen et al., 2010). Only a very few shared TCR sequences were identified between Treg and Tconv in one study of EAE in mice and none were identified when analysing an antigen specific TCR in the context of diabetes (Wong et al., 2007, Nguyen et al., 2010). This suggests that clonal relationships

between Tregs and other T cell lineages are extremely rare and may be difficult to identify.

Recently the microRNA, miR-10A has been identified as a possible regulator of the plasticity between Treg and T_{FH}. MiR-10A targets Bcl-6 and prevents the conversion of Treg to T_{FH} (Takahashi et al., 2012). This suggests that the conversion of Treg to T_{FH} is prevented.

Since the publication by Tsuji et al. (2009) describing the generation of T_{FH} from Tregs in CD3 ϵ ^{-/-} mice, an increasing body of evidence, including the findings in this chapter, suggests that this process does not occur in humans.

4.3.3 *Clonality between T follicular helper subsets*

When analysing part of the TCR β repertoire, related sequences were found in PCRs from CD57⁻ and CD57⁺ T_{FH}. This suggests that CD57⁺ and CD57⁻ T_{FH} are related developmentally as suggested in chapter 3. As related sequences are repeated over several different PCRs and therefore must be from different cells, this suggests that T_{FH} are clonally expanded which concurs with other studies (Golby et al., 1999).

Related sequences were also observed in both T_{FH} subsets and the control T cell subset. As the control population is not a distinct T cell lineage but is simply defined as a non-Treg population, it is possible that related T_{FH} are contained within the control population. On the other hand, the related sequences could be from another T cell lineage such as Th17 or Th1 that converted to T_{FH} or share a common progenitor (Lu et al., 2011). However additional sequence analysis of different T cell lineages would need to be done to explore this possibility further.

4.3.4 *Searching for clonality within a diverse population*

When analysing the TCR γ chain, a large amount of diversity was seen within the T cell subsets. Though clone specific primers were used to try to select for related clones between the subsets, sequencing of the PCR product showed that sequences of similar sizes were picked up but none were clonally identical.

Clonally related T cells may not have been detected due to the diversity of the sequences initially amplified with the TCR γ primers. However, when a specific family of TCR β genes was analyzed, two cases of clonality within the T_{FH} subsets were detected. This demonstrated that this is probably a better technique for analyzing clonality between T cell subsets. However still no clonality was found within the Treg population or between Tregs and T_{FH} when using a more specific method.

4.3.5 *Improving cell isolation methods*

If future studies were to continue to look for clonality between T_{FH} and Tregs by sequence analysis of the TCR, there are still improvements to be made in the isolation of T cell subsets. Similarities in surface antigen expression between T_{FH} cells and Tregs do pose a problem when looking at clonality. A contamination could give a false positive result. Recently a population of Treg cells have been described in GCs and termed follicular regulatory T cells (Tfr) (Linterman et al., 2011). These are most probably represented by the small percentage of foxp3⁺ cells in the CD127^{low} CD25^{high} PD-1⁺ gate (Fig 4.1C). Though as no clonality was found between Tregs and T_{FH} in this study, this could mean that the Tfr are not developmentally related to either Tregs or T_{FH} or any clonality was simply not detected. Separating Tfr from T_{FH} could be achieved by sorting foxp3⁺ T cells from the T_{FH} pool. However the intranuclear staining required to detect foxp3 could compromise the integrity of the DNA. This would be a challenge that would have to be overcome in future studies.

4.4 Conclusions

In this chapter several methods were designed for the detection of T cell subsets and clonally related cells. In conclusion:

- A new FACS gating strategy was established for isolating Treg subsets from lymphoid tissues.
- A difference in the frequency of naive and memory Tregs was observed between tonsil and blood.
- a method of amplifying and sequencing a fraction of the TCR β repertoire detected clonally related T cells, proving it as a viable method.
- No clonality was found between Treg and T_{FH} subsets from blood or lymphoid tissue.
- Two cases of clonality were found between and within T_{FH} subsets.

However, during the investigation, data on T_{FH} increased. The data in this chapter suggests no developmental relationship between Tregs and T_{FH} and this is consistent with the current literature. Rather than pursuing this further and confirming the identity of Tregs by, for example, functional analysis, a decision was made to move on and study T_{FH} and Tregs in colitis.

5 Chapter 5: Immune modulatory T cell subsets in Inflammatory Bowel Disease

5.1 Introduction

UC is an inflammatory disorder of the colonic mucosal layer. The inflammatory infiltrate includes an increase in Ig producing plasma cells, with the biggest relative increase being in cells secreting IgG1 (Scott et al., 1986).

Local production of autoantibodies has been identified in the mucosa in UC. Specificities include pANCA and tropomyosin 5 (Hibi et al., 1990, Targan et al., 1995, Onuma et al., 2000, Abad et al., 1997). However the exact role the autoantibodies play in the pathogenesis of the disease, if any, is not known. It is possible that they could be a consequence or cause in the disease process. Whatever their significance, the emergence of autoantibodies in UC suggests that T_{FH} may have a role, since T_{FH} are involved in regulating the specificity of B cell responses by affecting selection and can bias the B cell response against autoantibody production (Meyer-Hermann et al., 2006, Victora et al., 2010).

Features such as the production of autoantibodies, the predominant IgG1 response and the Th2 –like T-effector profile in UC lesions suggests that UC could be a humoral mediated disease (Fuss et al., 1996, Scott et al., 1986, Onuma et al., 2000). In contrast, there is a strong Th1 association and an overall pro-inflammatory phenotype in CD, which also results in a general increase in the production of IgG antibodies (Fuss et al., 1996, Scott et al., 1986). The effector B lineage cells of the gut are normally induced in the GALT (Brandtzaeg et al., 1999, Boursier et al., 2005). However the properties of immunoglobulins produced in diseased mucosa from UC patients have features

associated with a peripheral response (Thoree et al., 2002, Meenan et al., 1997).

SNPs in the *IL-2/IL-21* locus have been associated with UC (Festen et al., 2009). It is not known whether the SNPs reflect association of alleles of *IL-2* or *IL-21*. However enhanced IL-21 expression is found in inflamed mucosa from both UC and CD patients (Monteleone et al., 2005). IL-21 is an inflammatory cytokine but it is also important in the generation of T_{FH} and a mature immunoglobulin response and T_{FH} themselves produce a large amount of IL-21 (Ozaki et al., 2002, Vogelzang et al., 2008, Chtanova et al., 2004). IL-21 has been shown to be increased in autoimmune disease such as SLE and mouse models of lupus (Terrier et al., 2012, Linterman et al., 2009, Bubier et al., 2009, Ozaki et al., 2004). The SNP association at the IL-2/ IL-21 locus might reflect an involvement of T_{FH} in the pathogenesis of UC.

It has been suggested that the appendix could have a role in the pathogenesis of UC. Several studies found a significant negative correlation between appendectomy and the development of UC (Andersson et al., 2001, Naganuma et al., 2001). Comparisons between normal appendix and colon revealed that there is a trend towards a higher density of IgG+ cells in appendix (Bjerke et al., 1986). The appendix contains abundant GALT and dysregulation of the B cell response in the GALT of appendix might result in B cells homing to the colon and secreting proinflammatory complement fixing IgG autoantibodies that may contribute to the development of UC (Brandtzaeg, 2010, Halstensen et al., 1993).

There is a loss of immune regulation within the gut in IBD. Patients with immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome, which is caused by mutation in the Treg transcription factor, *FOXP3*, often have intestinal

inflammation similar to IBD (Gambineri et al., 2003). This is mirrored in mice lacking Treg derived IL-10, which have severe colitis due to uncontrolled immune responses to luminal antigens (Kühn et al., 1993). However, transfer of Tregs into CD4+CD45RB^{high} T cell induced mouse models of colitis slowed disease progression (Mottet et al., 2003). This demonstrated the importance of Tregs in controlling mucosal immune responses, suggesting that dysfunctional or reduced numbers of Tregs could be involved in the pathogenesis of IBD. However, the conclusions of previous studies on Tregs in IBD vary depending on how Tregs were identified and the controls used. Some studies have found an increase in intestinal Tregs in the lamina propria and mesenteric lymph nodes in CD compared to non-IBD controls and have demonstrated that they are functionally suppressive in vitro (Kelsen et al., 2005, Saruta et al., 2007). However a study in a SAMP1 / YitFc (SAMP) spontaneous mouse model of ileitis that is similar to human CD, concluded that Tregs are dysfunctional in vivo (Ishikawa et al., 2012). A study of Tregs in blood and colonic mucosa from patients with IBD or diverticulitis, used as an inflammatory disease control, demonstrated a reduced frequency of Treg in the blood of patients with active IBD compared to controls (Maul et al., 2005). Although the number of Tregs were higher in the mucosa in IBD compared to healthy controls, there were fewer Tregs in IBD than observed in diverticulitis (Maul et al., 2005). The lower number of Tregs in blood and mucosa in both active UC and active CD compared to inflammatory controls may be a consequence of apoptosis of Treg in IBD (Maul et al., 2005, Veltkamp et al., 2011).

T_{FH} in IBD have not been studied in detail. It is possible that an equilibrium between immune regulators such as T_{FH} and Tregs is important for immune homeostasis and that this becomes unbalanced in IBD. The appendix tissue is thought to be involved in the development of UC and in this chapter T_{FH} and Treg subsets in IBD, control tissue and

peripheral blood were analysed by immunohistochemistry and flow cytometry and in particular comparisons between UC appendix and other intestinal mucosa were made.

5.1.1 ***Aims:***

- To determine if there is any difference in the frequency or phenotypic diversity of T_{FH} in UC compared to controls that might reflect a role of T_{FH} in the generation of autoantibodies and inflammation in UC.
- To determine if there is any difference in the frequency of circulating T_{FH} in IBD compared to healthy controls.
- To determine if there is any difference in the frequency of mucosal and circulating Tregs in UC compared to controls that may be related to the dysregulation of humoral immune responses.

5.2 ***T_{FH} subsets in inflammatory bowel disease.***

5.2.1 ***T_{FH} frequency in the IBD gut tissue***

As T_{FH} are involved in the selection and generation of plasma cells through a GC response, it is possible that an altered T_{FH} population contributes to the altered humoral pathology seen in UC. In order to study T_{FH} in IBD paraffin embedded gut tissue from bowel cancer, UC and CD patients who underwent a right hemicolectomy, were obtained. Blocks of control normal ileum, appendix and colon were selected from uninvolved areas of right hemicolectomy specimens from patients undergoing resection for bowel cancer. Blocks of ileum, appendix and colon in which the tissue was involved in the disease but where tissue architecture was not obscured by ulceration or fibrosis, were selected from the UC and CD resections.

To identify and analyse GCs in tissue sections from all the tissue types described above, sections were stained for CD21, which is expressed by follicular dendritic cells.

Differences in GC size were determined with the use of a microscope eye piece graticule and the average size of GCs in a tissue block was plotted.

In UC, GCs in the appendix were significantly smaller compared to those in cancer appendix (Fig 5.1A and B). GCs in UC ileum and colon also tended to be smaller than in control tissue but the difference was not significant.

Next, serial tissue sections from each tissue block were double stained for CD57 and CD3. The relative T_{FH} density was determined by the same method as described in 3.2.3. The number of CD57+ cells, CD3+ cells and haematoxylin only stained cells within GCs were counted and expressed as a ratio of T_{FH} to non-T cells and a relative T_{FH} density was generated.

A significantly higher relative T_{FH} density was observed in UC appendix GCs compared to cancer appendix GCs (Fig 5.1 A and C). However the relative T_{FH} density in UC ileum and colon GCs was comparable between cancer ileum and colon GCs and there was no significant difference in the relative density of T_{FH} in CD gut tissue GCs compared to cancer gut tissue GCs (Fig 5.1C).

The average relative density of CD57+ T_{FH} in UC appendix GCs was significantly higher than in cancer appendix GCs but there were no other differences between the tissue types tested (Fig 5.1C). There was a trend for the average relative density of CD57- T_{FH} in UC appendix to be lower than in control tissues but there were no significant differences as the average relative density of CD57- T_{FH} was diverse within data sets (Fig 5.1C).

Overall this data suggests that even though the GCs in UC appendix are small there is a high relative density of T_{FH} .

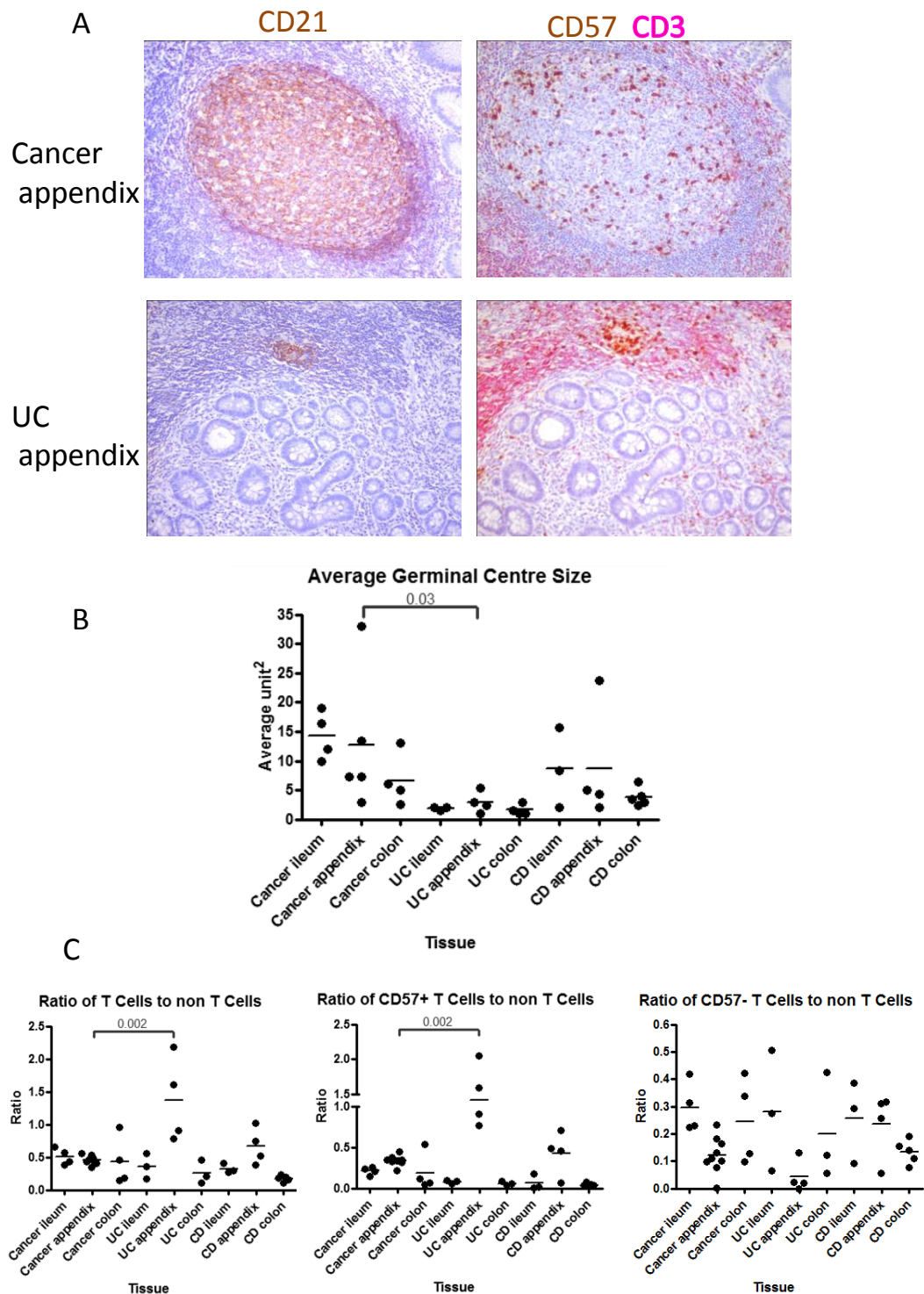


Figure 5-1 GC size and T_{FH} ratio in cancer, UC and CD gut tissue.

A- Paraffin embedded serial tissue sections from cancer and UC appendix IHC stained for CD21 (left) and CD57 in brown and CD3 in pink(right). B-The average area of >3 GC on each tissue section calculated by the number of 1mm² filled by the CD21+ area on a 10mm x 10mm graticule at 10x magnification. C- The average ratio of all CD57+ and CD3+ T cells to non T cells in GCs (left). The

average ratio of CD57+ T cells to non T cells within GCs (middle). The average ratio of CD3+ only T cells to non T cells within GCs (right). Mann Whitney statistical test performed on B and C.

The relative density of T_{FH} was high in tissue sections of UC appendix, but in order to study the overall frequency of T_{FH} , fresh appendix cell suspension from cancer, UC and CD patients was analysed by flow cytometry. T_{FH} were identified as CD3+ CD4+ CXCR5^{high} PD-1^{high} and the frequency of CD57+ T_{FH} within this gate was also analysed.

There was no significant difference between the frequency of T_{FH} in CD compared to controls. Statistical tests could not be performed for the UC data set due to the low number data points. The average percentage of T_{FH} in UC was 5.5% compared to an average of 3.9% in control appendix (5.2A). There were no differences in the frequencies of CD57+ T_{FH} between control, UC and CD appendix (5.2 B).

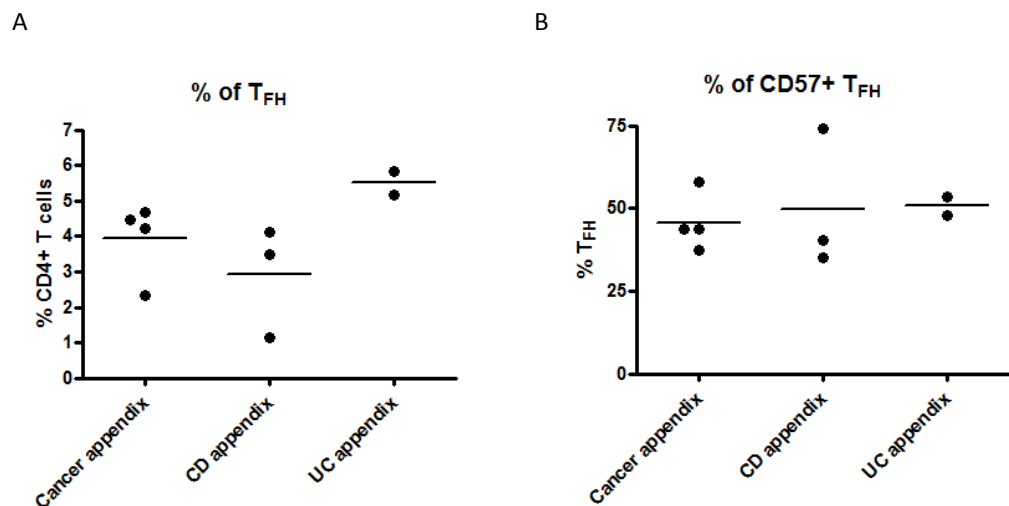


Figure 5-2 FACS analysis of T_{FH} frequencies in cancer, UC and CD appendix

Fresh appendix mononuclear cells from cancer, UC and CD Appendix analysed for T_{FH} markers. A- Percentage of $CXCR5^{high}PD-1^{high}$ T_{FH} in $CD4^{+}$ T cell population. B- Percentage of $CD57^{+}$ T_{FH} in the $CXCR5^{high}PD-1^{high}$ T_{FH} population. Mann-Whitney statistical test performed on A and B.

5.3 Identification of circulating T_{FH} cells

In Chapter 3 T_{FH} were analysed in lymphoid tissues. In this chapter potentially equivalent populations were sought in blood.

Subsets of circulating ‘ T_{FH} -like’ cells have been described by several different groups and therefore termed circulating T_{FH} (cT_{FH}) (Simpson et al., 2010, Morita et al., 2011). As different subset ratios of T_{FH} were observed in UC tissue compared to cancer tissue, the frequency of cT_{FH} in IBD and healthy controls was analysed.

The phenotype of cT_{FH} was found to be very different to T_{FH} in lymphoid tissues. Both circulating and tissue T_{FH} can be identified by their high expression of $CXCR5$. In lymphoid tissue there is dual expression of $CD57$ with $CXCR5$ on $CD4$ T cells, but on circulating $CD4$ T cells $CD57$ is expressed mainly on $CXCR5^{-}$ populations (Fig 5.3A). An antibody panel including $CXCR5$ and $PD-1$ was used to identify cT_{FH} in this study as this is most commonly used (Bossaller et al., 2006).

The expression levels of $PD-1$ staining on $CXCR5^{high}$ cells was lower on circulating $CD4$ T cells than in lymphoid tissue, where T_{FH} are $PD-1^{high}$ (Fig5.3A and B). In figure 5.3A, the smaller box shows the gating for $CXCR5^{high}PD-1^{+++}$ used to identify T_{FH} in lymphoid tissue (Yu and Vinuesa, 2010). However as cT_{FH} express lower levels of $PD-1$, they are identified as $CXCR5^{high}PD-1^{++}$ cells and lie within the larger box in figure 5.3A. Circulating T_{FH} in healthy, UC and CD blood was analysed using the gating for $CXCR5^{high}PD-1^{++}$ T cells, as described by others (Simpson et al., 2010).

Patients with active IBD were separated from patient in remission or with inactive disease. A significantly higher frequency of cT_{FH} was observed in patients with active CD compared to healthy controls (Fig 5.3C). However the frequency of cT_{FH} within this data set is highly variable. Analysis of cT_{FH} and the patients' age, gender and medication within the active CD group showed no association between frequency of cT_{FH} with any of these factors.

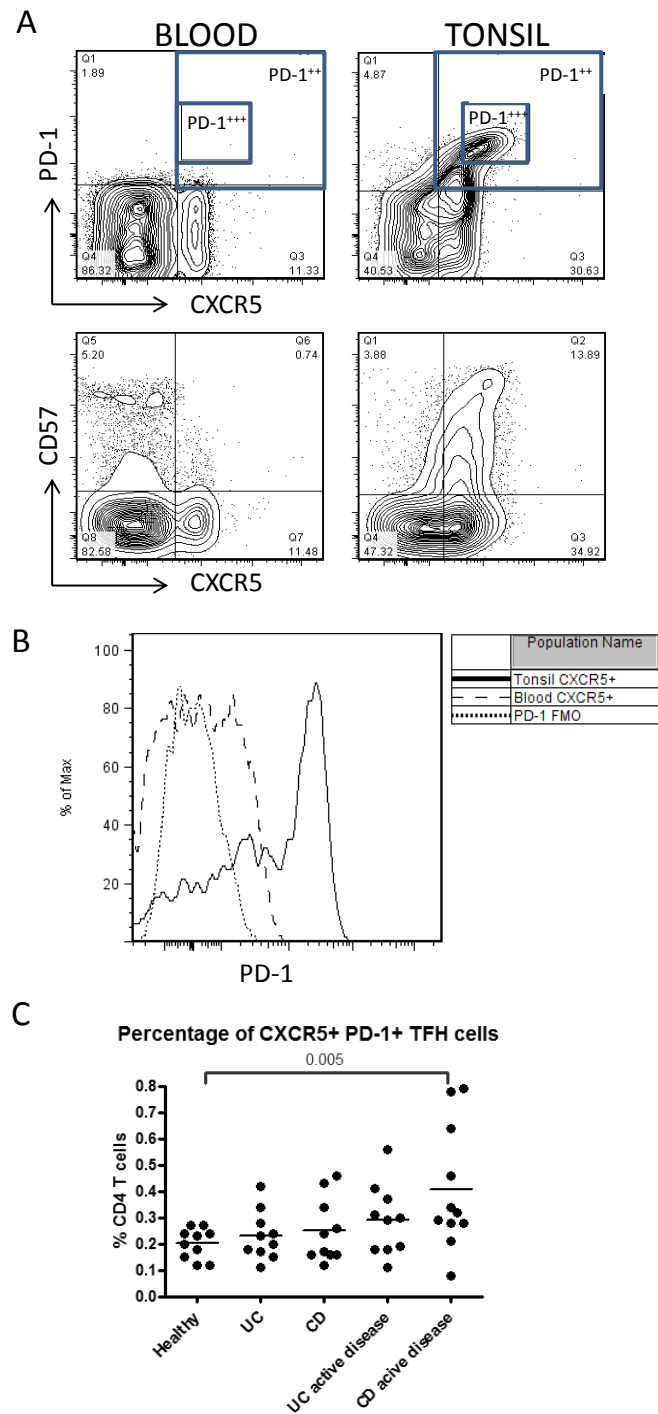


Figure 5-3 Frequency of T_{FH} in peripheral blood of patients with IBD

Circulating T_{FH} cells and associated co-stimulatory markers analysed by flow cytometry. A-B Paired blood and tonsil mononuclear cells analysed for T_{FH} associated markers by flow cytometry. Gating on CD3+CD4+ T cells prior to FACS plots shown. Top left and right plot- expression of PD-1 and CXCR5 on blood and tonsil T cells. Bottom left and right plot-expression of CD57 and CXCR5 on blood and tonsil T cells B- Histogram of PD-1 expression on CXCR5+ cells, previously gated on CD3+CD4+ T cells in an example of paired tonsil and blood. C- percentage of PD-1+CXCR5+ T_{FH} in CD4 T cell population from healthy controls (n=10), inactive UC (n=10), inactive CD (n=10), active UC (n=10) and active CD (n=11) as defined by quadrant 2 on FACS plot A top left. Mann Whitney statistical test performed on C.

5.2.3 CD40L expression in inflammatory bowel disease

CD40L is induced on T cells by activation and is essential for GC formation and the survival of B cells in a GC (Quezada et al., 2004). Therefore the frequency of CD4 T cells with surface CD40L was analysed by flow cytometry (Fig 5.4A).

There was no difference in the frequency of CD40L+ T cells between any groups analyzed apart from in blood from patients with active CD compared to healthy controls (Fig 5.4B). However this apparent increase in the frequency of CD40L+ T cells in CD compared to healthy controls is due to one particularly high data point. Using the Grubbs test, the point circled in figure 5.4B was identified as a significant outlier. When this point is excluded there is no longer a significant difference and therefore there is probably no true biological difference between these two data sets (Fig 5.4C).

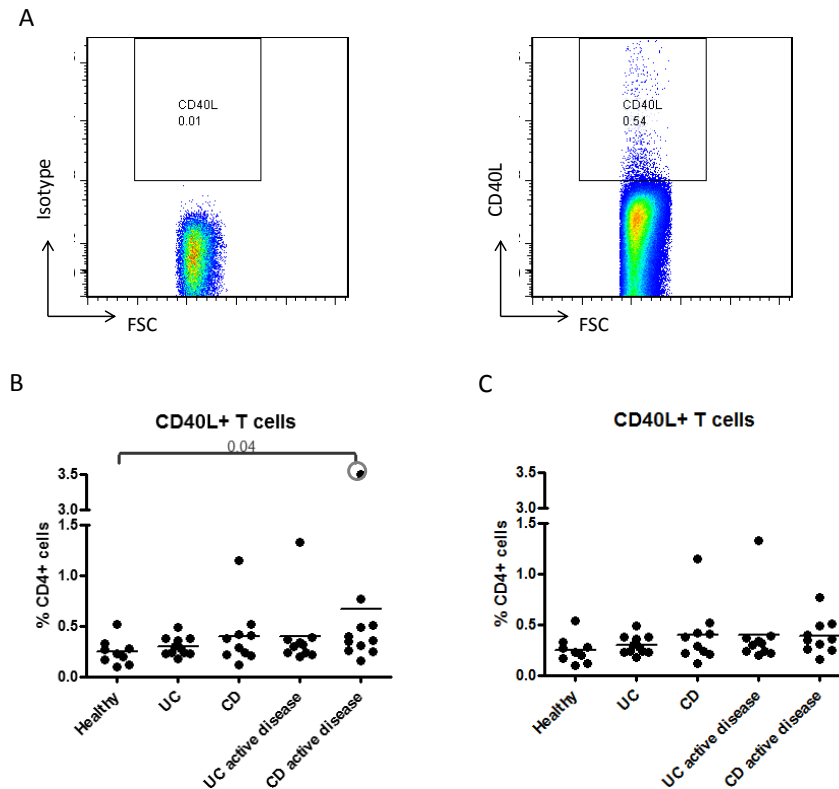


Figure 5-4 Frequency of CD40L+ CD4 T cells in peripheral blood of patients with IBD

Analysis of CD40L on CD4 T cells in PBMCs from healthy controls and IBD patients by FACS A- Representative FACS plots with prior gating on CD3+CD4+T cells of healthy blood isotype (left) and CD40L (right). B- Percentage of CD40L+ cells within CD4+ T cells from the blood of healthy controls (n=9), inactive UC (n=11) inactive CD (n=10), active UC (n=10) and active CD (n=11) patients. C- Graph in B without the data point circled. Mann Whitney statistical test performed on B and C.

5.3 Analysis of T_{FH} associated molecules

5.3.1 Expression of CXCL13 in IBD gut tissue.

FDC and germinal centre stromal cells secrete CXCL13 that attracts B cells and T cells expressing CXCR5 (Cyster et al., 2000). Altered CXCL13 expression might be associated with the size of GCs by altering the recruitment of cells (Marchesi et al., 2009). To investigate if CXCL13 expression contributes to the distorted GC phenotype observed in UC, tissue sections of ileum, appendix and colon from cancer and IBD patients were stained for CXCL13 by IHC. The average number of CXCL13+ cells

within the GC, lymphocytic infiltrate and LP was determined by counting cells in a defined area using an eyepiece graticule, a figure considered to reflect cell density.

The average density of CXCL13+ cells was highly variable within all data sets (Fig 5.5). There was no significant difference in the average number of CXCL13+ cells in the GC or lymphocytic infiltrate between UC, CD and cancer tissue (Fig 5.5A and B). However as the data sets were small and there was a wide spread of data within them, it is possible that the variability might mask any genuine biological differences. However, a significant difference in the density of CXCL13+ cells per high power field was observed between UC appendix LP and LP in UC colon and ileum (Fig 5.5C). As the data set was small, this difference would have to be verified using further samples, but unfortunately no more were available for this study.

5.3.2 ***IL-17+ cells in all gut tissue microenvironments.***

In figure 3.6 it was demonstrated by flow cytometry that T_{FH} can produce IL-17 and IL-17+ cells were identified in GCs by IHC. Excess IL-17 production has been associated with the development of autoantibodies, inflammation, and the production and secretion of mucosal antibodies (Nakae et al., 2003, Hofstetter et al., 2005, Cao et al., 2012). Therefore the expression of IL-17 in ileum, appendix and colon tissue from IBD patients and controls was assessed by IHC, and the number of cells per high power field in different microenvironments was determined.

Very few IL-17+ cells were identified in any of the tissues tested (Fig 5.6) IL-17+ cells were identified in some GCs but there was no significant difference between gut tissue from UC, CD and cancer patients (Fig 5.6A).

There was a trend towards a higher number of IL-17+ cells in the CD appendix LP but there was only a significant difference between CD appendix and CD colon (Fig 5.6C).

In one case of CD the number of IL-17+ cells in the appendix was high in GCs, lymphocytic infiltrate and the LP (Fig 5.6A, B and C). The significance of this is not known.

5.4 *Regulatory T cells in Inflammatory Bowel Disease*

5.4.1 *Regulatory T cells in IBD gut tissue*

To investigate the overall frequency of Tregs in IBD gut tissue, the percentage of CD127^{low}CD25^{high}PD-1^{low} Tregs were analysed in cancer, UC and CD appendix cell suspensions by flow cytometry using the parameters described in figure 4.2.

Due to small data sets, statistical analysis could not be performed on the UC appendix data. However there was no significant difference in the overall frequency of Tregs within the CD4 T cell population between cancer and CD appendix (Fig 5.7A). Analysis of the naive, memory and activated Treg subsets also showed no significant differences in these subsets between cancer and CD appendix (Fig 5.7B).

Therefore overall no difference in Treg frequency was observed between cancer, CD and UC appendix tissue by FACS analysis.

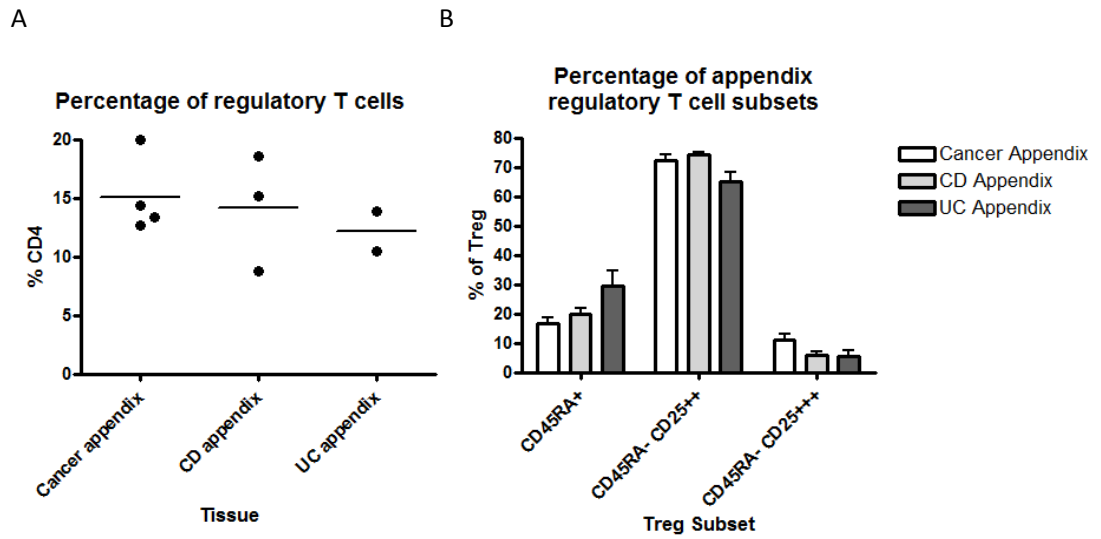


Figure 5-7 Frequency of Tregs in cancer, UC and CD appendix.

Fresh appendix mononuclear cells from cancer (n=4), CD (n=3) and UC (n=2) patients were analysed for Treg markers by FACS. A- Percentage of CD127^{low}CD25^{high}PD-1^{low} Tregs in CD4⁺ T cells. B- Percentage of naive CD45RA⁺ Tregs, memory CD45RA-CD25⁺⁺ Tregs and activated CD45RA-CD25⁺⁺⁺ Tregs within the Treg population. Mann Whitney statistical test performed on A and B.

To investigate whether Treg frequency changes in different areas of the gut or different microenvironments in IBD, tissue sections of ileum, appendix and colon from cancer, UC and CD patients were stained by IHC to detect the Treg transcription factor, foxp3. The number of foxp3⁺ cells per field was counted in 3 different gut micro-environments; germinal centres, lymphocytic infiltrate and LP.

There were no significant differences in the frequency of foxp3⁺ cells in the T cell population between control, UC and CD tissue in any of the micro-environments (Fig 5.8). However a significantly higher proportion of foxp3⁺ cells was observed in the

lymphocyte infiltrate and LP in control appendix compared to the cancer ileum and colon (Fig 5.8B and C). The proportion of foxp3+ cells was also significantly higher in the lymphocytic infiltrates of the appendix compared to the lymphocytic infiltrates of the ileum and colon in UC patients (Fig 5.8B). A similar trend of a higher frequency of foxp3+ T cells in the appendix compared to the ileum and colon was seen in the LP of patients with UC and CD but the difference was not significant (Fig 5.8C).

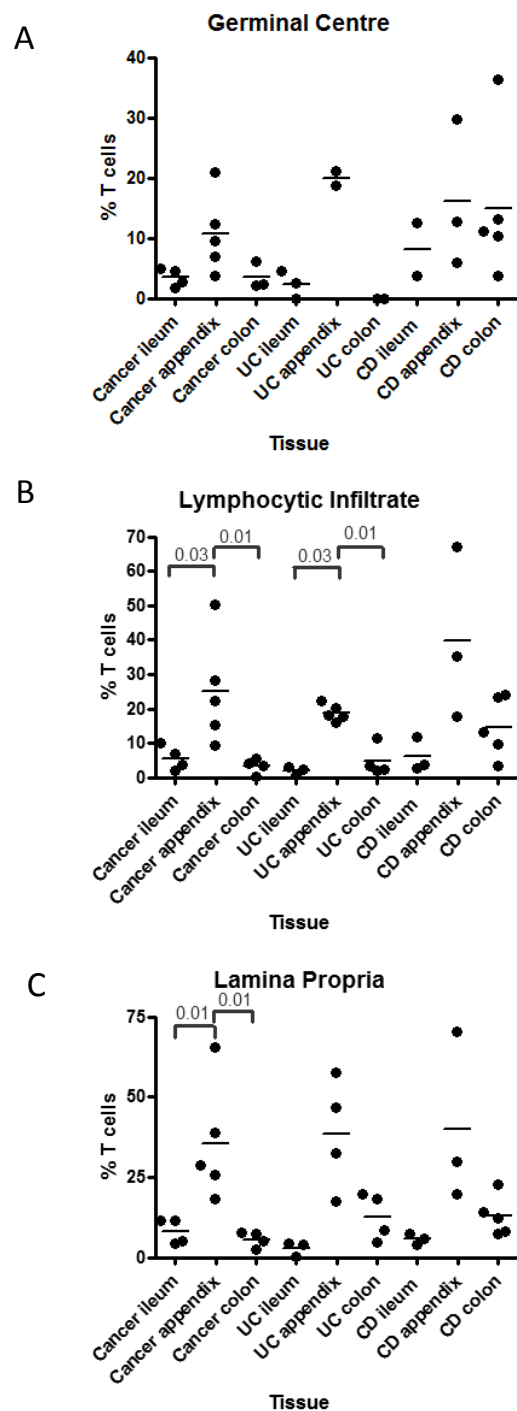


Figure 5-8 IHC analysis of Foxp3+ T cells in cancer and IBD gut

Paraffin embedded sections of ileum, appendix and colon from cancer, UC and CD patients single stained for foxp3 and CD3. A- Percentage of foxp3+ cells T cells within GCs, B- Percentage of foxp3+ cells T cells at x60 magnification in 10mmx10mm within lymphocyte infiltrate C- Percentage of foxp3+ cells T cells at x60 magnification in 10mmx10mm within lamina propria. Mann-Whitney statistical test performed on A, B and C.

5.4.2 *Circulating Subsets of Regulatory T cells in Inflammatory Bowel Disease*

Previous studies have reported a decrease in CD25^{high} circulating T regs in active IBD compared to inactive IBD (Maul et al., 2005). Since these studies, CD127 has been introduced alongside CD25 to more accurately identify Treg (Liu et al., 2006). Therefore analysis of circulating CD127^{low}CD25^{high} Tregs in active and inactive UC and CD and healthy controls by flow cytometry was performed.

There was a trend towards a lower percentage of circulating CD127^{low}CD25^{high} Treg within the CD4+ T cell pool in patients with active UC compared to healthy controls that nearly reached statistical significance (p 0.052) (Fig 5.9A and C). However there was no difference in the percentage of Tregs in active CD compared to inactive CD or healthy controls (Fig 5.9A and C).

Naive Tregs can be separated from activated/memory Treg by their expression of CD45RA (Miyara et al., 2009). In active UC the percentage of naïve cells (CD45RA+) within the CD127^{low}CD25^{high} Treg gate was higher than healthy controls and inactive UC (Fig 5.9B and D). However when the percentages of CD45RA+ and CD45RA- Tregs were analysed within the whole CD4+ T cell population it is apparent that the CD45RA- Tregs were significantly decreased in active UC and the percentages of

CD45RA⁺ Tregs were equivalent to that found in inactive UC and healthy controls (Fig 5.9E and F).

The transcription factor, foxp3, can also be used to identify Tregs (Hori et al., 2003). There was no difference between the percentage of CD127^{low}CD25^{high} and Foxp3⁺CD25^{high} Tregs in any patient groups (Fig 5.10A).

Foxp3 expression within CD127^{low}CD25^{high} CD4 T cells and the CD45RA⁺ and CD45RA⁻ subsets was assessed by flow cytometry. Approximately 80% of CD25^{high}CD127^{low} Tregs are foxp3⁺ in all patient groups consistent with the hypothesis that the majority of T cells found within these gates are Tregs (Fig 5.10B).

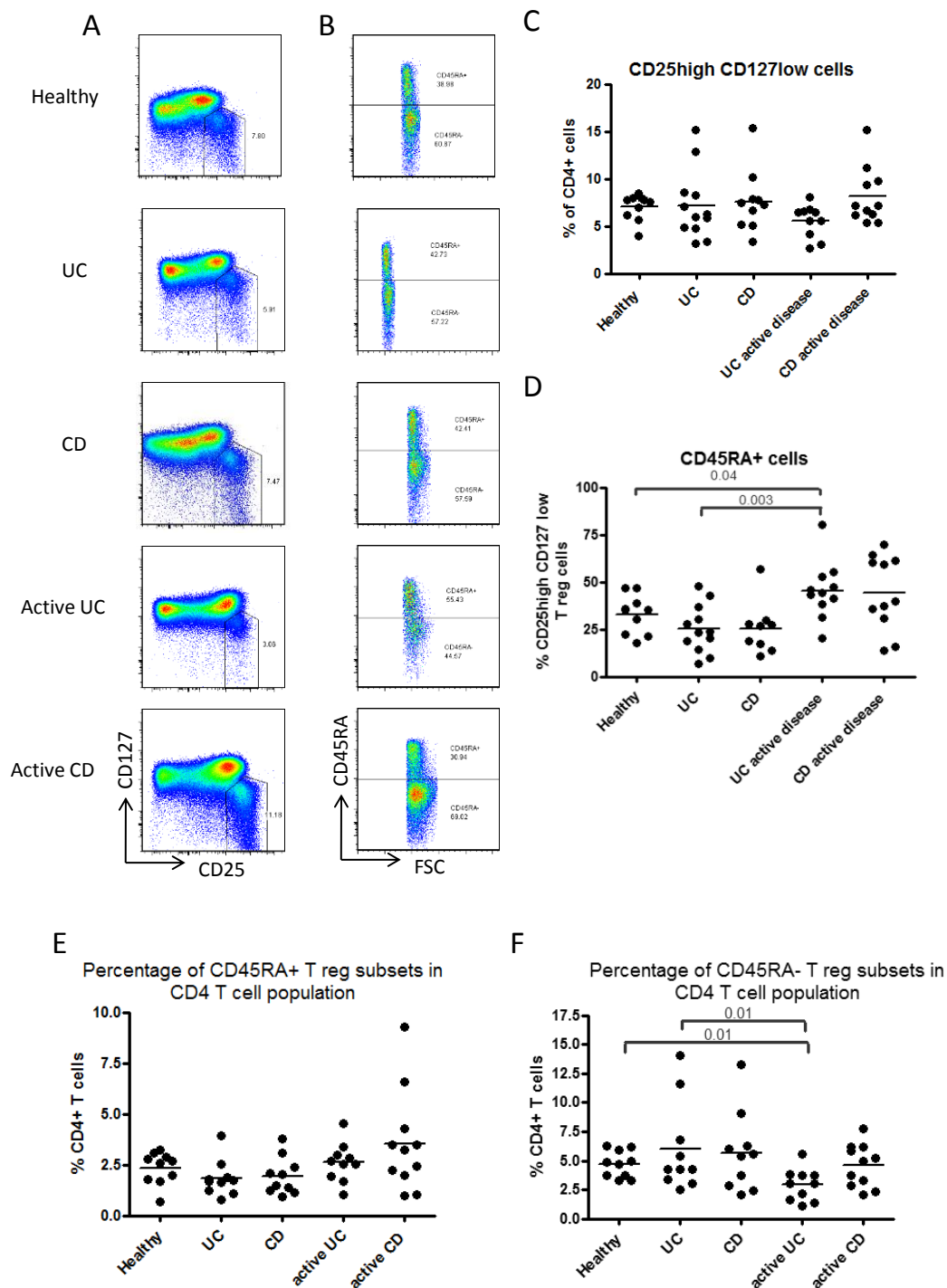


Figure 5-9 Analysis of circulating T reg subsets in IBD by flow cytometry

PBMCs from healthy control, patients with inactive UC and CD and patients with active UC and CD were analysed for Treg markers by flow cytometry. An example of gating for CD127^{low}CD25^{high} Tregs in the CD4 population (A and C) and the frequency of Tregs in the CD4 population (B and D) in healthy controls (n=10), inactive UC (n=10), inactive CD (n=10), active UC (n=10) and active CD (n=11). C- An example of gating for separating CD45RA⁺ and CD45RA⁻ Tregs in the Treg population (left) and the frequency of CD45RA⁺ in the Treg population (right) in healthy controls (n=10), inactive UC (n=10),

inactive CD (n=10), active UC (n=10) and active CD (n=11). Percentage of CD45RA+ (E) and CD45RA- Tregs (F) in the CD4 population in healthy controls (n=10), inactive UC (n=10), inactive CD (n=10), active UC (n=10) and active CD (n=11) Mann Whitney statistical test performed in C, D, E and F.

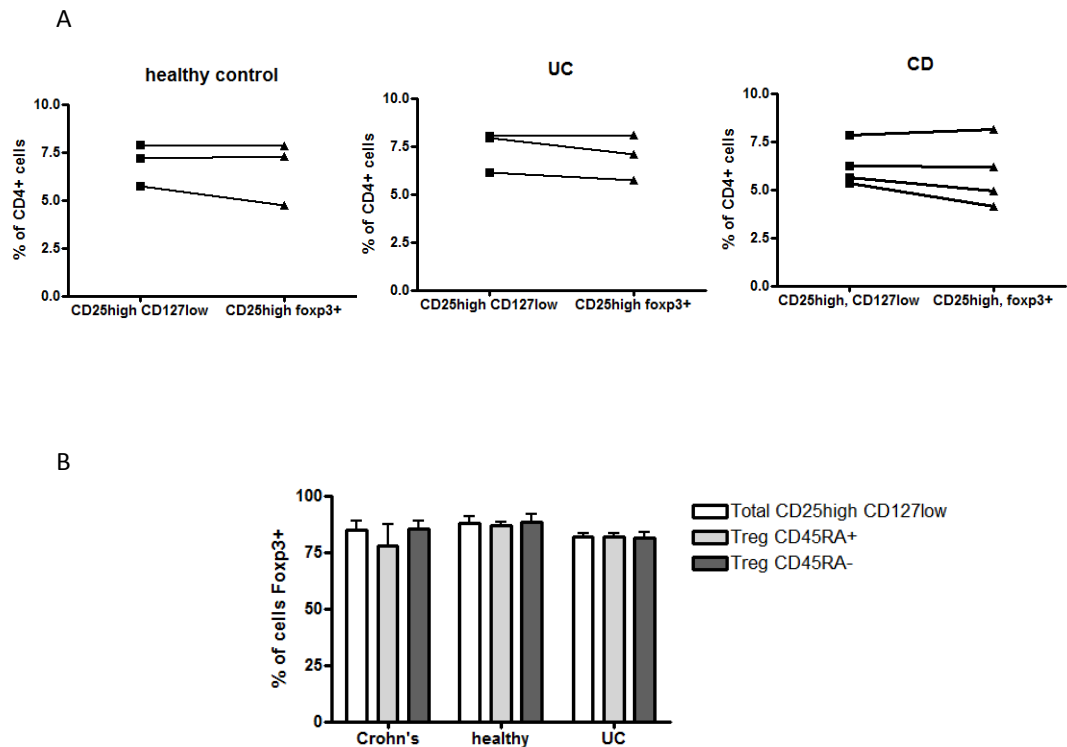


Figure 5-10 Analysis of foxp3 expression in circulating Treg subsets in IBD

A- Comparison of CD127^{low}CD25^{high} cells and foxp3+CD25^{high} cells frequency in the CD4 T cell population in healthy controls (n=3), UC (n=3) and CD (n=4) patients. B- Percentage of foxp3+ cells with CD127^{low}CD25^{high} cells, CD45RA+ Tregs and CD45RA- Tregs from healthy controls (n=3), CD (n=4) and UC (n=3) patients. Wilcoxon matched pairs statistical test performed in A.

The CD45RA- population includes memory and activated Tregs that can be separated by their expression of CD25 (Miyara et al., 2009). Memory Tregs are CD45RA-CD25⁺⁺ and activated Tregs are CD45RA-CD25⁺⁺⁺. The frequency of these two Treg subsets within the Treg population was analysed by flow cytometry.

The frequency of circulating memory Tregs was significantly lower in active UC compared to inactive UC and healthy controls (Fig 5.11A and B). The frequency of

activated Tregs was also significantly lower in the blood of patients with active UC compared to inactive UC and healthy controls (Fig 5.11A and C). However there was no significant difference in the frequency of circulating Treg subsets between UC and CD patients possibly due to patients with CD having diverse and varied Treg frequencies (Fig 5.11B and C).

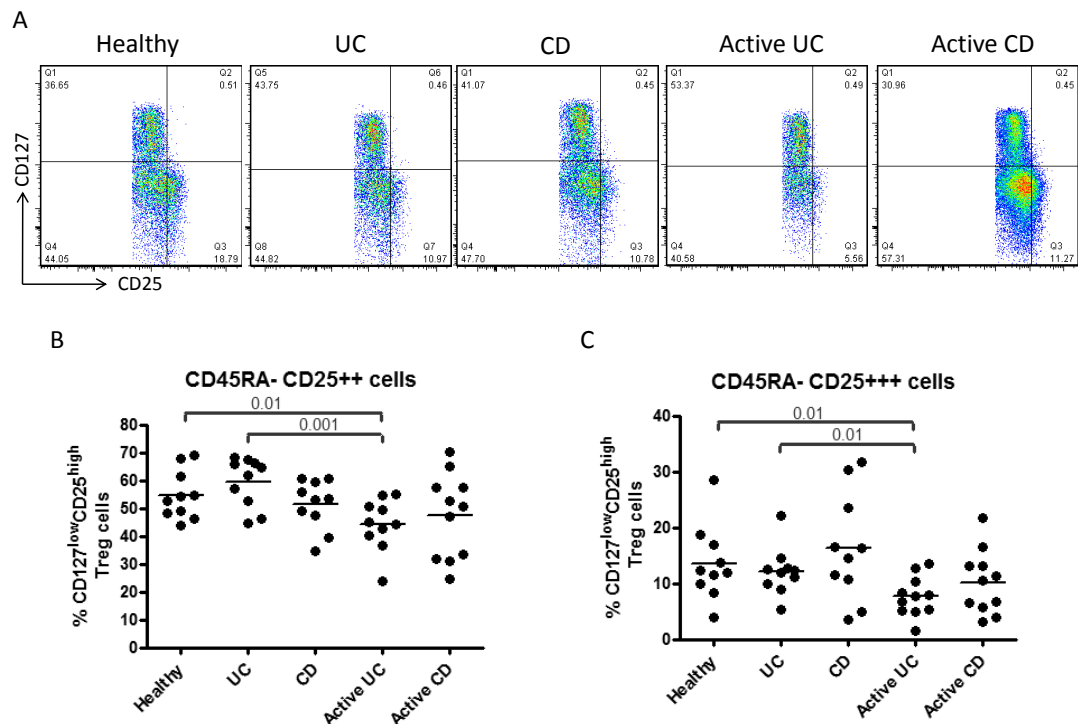


Figure 5-11 Analysis of activated and memory Treg subsets

Analysis of CD45RA- Treg subsets within the CD127^{low}CD25^{high} Treg population by flow cytometry in healthy controls, inactive UC and CD patients and active UC and CD patients PBMCs. A- An example of separating naive CD45RA+ Tregs, memory CD45RA-CD25++ Tregs and active CD45RA-CD25+++ Tregs within the CD127^{low} CD25^{high} Treg population by FACS in healthy controls, inactive UC, inactive CD, active UC and active CD. B Percentage of , memory CD45RA-CD25++ Tregs within the CD127^{low} CD25^{high} Treg population in healthy controls (n=10), inactive UC (n=10), inactive CD (n=10), active UC (n=10) and active CD (n=11). C- active CD45RA-CD25+++ Tregs within the CD127^{low} CD25^{high} Treg population in healthy controls (n=10), inactive UC (n=10), inactive CD (n=10), active UC (n=10) and active CD (n=11).

5.5 Discussion

5.5.1 Role of the appendix in UC

In this chapter differences were observed in UC appendix compared to other UC gut mucosa and to cancer and CD tissue.

- GCs in UC appendix were significantly smaller than GCs in cancer appendix, or GCs in UC mucosa outside the appendix.
- A higher relative density of T_{FH} was seen in appendix GCs in UC compared to appendix GCs in cancer and CD or in GCs in other UC mucosa.
- T_{FH} in appendix GCs in UC were mainly CD57+, whereas GCs in the ileum and colon in UC contained a substantial proportion of CD57- T_{FH} like in CD and cancer tissue.

In addition to these differences in GCs in UC appendix compared to GC's in other tissues and GCs in appendix in other conditions, more CXCL13+ cells were observed in the UC appendix LP compared to UC colon and ileum LP. Though CXCL13+ cells have not been reported in the LP of healthy or IBD tissue, Marchesi et al. (2009) suggest that CXCL13 within the effector tissue of the gut may recruit lymphocytes during inflammation and increase lymphoid tissue formation. Also, in general, a higher frequency of Tregs was identified in appendix compared to other mucosal sites.

An association between the appendix and UC has been demonstrated by several studies that found a negative correlation between appendectomy and disease development. Large cohort studies show that patients who had appendectomies due to non-perforating appendicitis before the age of 20 were less likely to develop UC compared to controls (Andersson et al., 2001, Naganuma et al., 2001). Several studies have compared the appendectomy rate in CD patients to control groups. However, the

majority of studies have not found a significant correlation but this may be due to CD patients undergoing appendectomies at the onset of disease before or at the time of diagnosis (Radford-Smith et al., 2002, Radford-Smith, 2008).

Radford-Smith et al. (2002) suggested two hypotheses to explain why the removal of the appendix may protect against the onset of UC. Firstly Radford-Smith et al. (2002) proposed that individuals who undergo an appendectomy may be genetically different or are under the influence of different environmental factors to individuals that develop UC. For example, patients than undergo appendectomies may be predisposed to developing appendicitis whereas patients with UC are not predisposed to appendicitis but are predisposed to developing UC. Secondly Radford-Smith et al. (2002) proposed that removal of the appendix may affect immune modulation of the gut mucosa.

In TCR α mutant mice that spontaneously develop an inflammatory disease like UC, fewer mice developed IBD over the 6-7 months of observation after removing the appendix at 1 month old (Mizoguchi et al., 1996). This data would suggest that removing the appendix at an early age modifies the gut immune responses and supports Radford-Smith et al. (2002) hypotheses. However, though mouse models give an insight into the disease progression of UC-like disease after an appendectomy, there are several differences in the mouse and human appendix such as size and possibly function. Therefore direct comparisons cannot be made between mouse models and human disease.

The data in this thesis might support both hypotheses of Radford-Smith et al. (2002). On the one hand, the distinctive properties of the GCs in the appendix in UC implies that they may have encountered an environmental pathogen that initiated a distinctive immune response, or that the response in appendix of patients with UC might be genetically different so that GCs in appendix in patients with UC develop differently. On the other hand, the higher frequency of Treg in the appendix in general, compared to

other sites in the gut, suggests that appendix may have a unique role in maintaining mucosal immune homeostasis therefore that removal of the appendix may cause immune modulation in the intestine that could protect against UC. Subsequent likely events are discussed in the paragraphs below.

5.5.2 *Possible support of autoantibody production by T_{FH} in UC*

A very high relative density of T_{FH} was seen exclusively in appendix GCs in UC, when analysed by IHC. A high density of T_{FH} has been proposed as a mechanism for supporting the development of autoreactive B cells (Linterman et al., 2009). A higher frequency of T_{FH} could theoretically reduce the competition between GC B cells for T_{FH} interactions and cytokines (Meyer-Hermann et al., 2006). Therefore B cells that would otherwise die due to selective pressure may instead receive the signals needed to survive. Limiting T_{FH} numbers usually ensures that B cells specific for the antigen mature however an excess of T_{FH} would allow less antigen specific B cells to survive and these could be autoreactive (Linterman et al., 2011). Several different autoreactive antibodies have been associated with UC and the high density of T_{FH} observed in UC may contribute to the local production of autoreactive B cells (Onuma et al., 2000, Targan et al., 1995).

In TCR α mutant mice that develop UC-like disease, an increase in autoantibodies similar to those found in UC were found in the appendix lymphoid tissue compared to Peyer's patches of these mice (Mizoguchi et al., 1996). In this study a high relative density of T_{FH} was only observed in the appendix lymphoid tissue in UC which suggests that appendix GCs could be an early induction site of autoantibodies in UC. This is consistent with the hypothesis that the appendix is involved in the development of UC

and removing the appendix modulates the mucosal immune system which prevents the onset of UC (Radford-Smith et al., 2002).

5.5.2.1 *T_{FH} phenotype in UC appendix*

The frequency of CD57+ T_{FH} obtained by FACS analysis shows no differences between cancer, UC and CD appendix. However a difference in the relative density of CD57+ T_{FH} was observed between UC and cancer appendix by IHC. This is probably due to FACS being able to detect non T_{FH}, such as activated T cells which also express CXCR5 that localise in gut LP (Sarraf et al., 2010a). Therefore it is possible that an increase in activated T cells in UC masks any difference in the frequency of CD57+ T_{FH} when analysing by flow cytometry.

Analysis of T_{FH} by IHC shows that T_{FH} in UC appendix were mainly CD57+ whereas CD and cancer appendix contained a substantial proportion of CD57- T_{FH}. The high relative density of CD57+ T_{FH} observed in the UC appendix resembles the phenotype of T_{FH} in peripheral rather than mucosal lymphoid tissues (Fig 3.2 and Fig 5.1).

An increase in the production of IgG, in particular IgG1, is associated with UC and this isotype is more commonly induced in peripheral lymphoid tissues (Scott et al., 1986). Also IgHJ1 usage was detected in the UC mucosal IgG population and IgHJ1 is associated with the peripheral immunoglobulin repertoire (Thoree et al., 2002). It is possible that in UC appendix the high proportion of CD57+ T_{FH} may support CSR to IgG and a peripheral-like GC response.

Brandtzaeg (1995) also suggests that other features of IBD reflect that of the peripheral immune system rather than the mucosa immune system. Vascular endothelial cells in IBD mucosal tissue express adhesion molecules similar to those found in peripheral lymphoid tissue and gut derived lymphocytes in IBD have the ability to bind

to peripheral vessels (Salmi and Jalkanen, 2001, Salmi et al., 1994). Normally intestinal T cells are unreactive to certain microbial antigens but primed T cells from the mucosa of IBD patients having similar microbial specificities as those in the periphery (Pirzer et al., 1991). As the gut is a specialised microenvironment where immune cells are in close proximity to the luminal pathogens, strict regulation of responses and minimal inflammatory responses are needed to maintain the integrity of the gut (Barnes and Powrie, 2009). Therefore if an immune response similar to a peripheral immune response occurs in the gut it could result in mucosal inflammation.

It has been suggested by Brandtzaeg (2010) that the change in immunoglobulin profile might be a defence mechanism. Secretory IgA is a first line defence mechanism, which mediates immune exclusion (Mantis et al., 2011). In inflammatory bowel disease, there is an increase in IgA⁺ plasma cells, but fewer IgA express the J chain which result in less IgA being secreted into the lumen (Brandtzaeg, 2010, Kett et al., 1988). This could lead to a reduction in the first line defence mechanisms. However IgG is increased, possibly as less IgA is secreted into the lumen and gut microbial bacteria are not sufficiently controlled. The IgG in IBD possibly acts as a second defence mechanism when the immune system has already been compromised as IgG is efficient at opsonising pathogens and activating complement (Brandtzaeg, 2010). The difference in T_{FH} could be a result of the need to switch from sIgA to IgG due to the breaching of the intestinal defences. A change in T_{FH} phenotype could promote movement towards a more systemic type response or it could be associated with a reduction in sIgA that might subsequently result in an increase in IgG which then compromises the mucosal defences (Macpherson et al., 2008).

The high proportion of CD57⁺ T_{FH} was observed exclusively in UC appendix and not in the colon or ileum. This suggests the appendix may be the origin of the

peripheral-like response in UC, predominantly a disease of the colon. If so, this would support Radford-Smith et al. (2002) hypothesis that the appendix is able to modify the gut responses.

5.5.2.2 Higher frequency of Treg in appendix tissue

In both cancer and UC tissue there was a higher frequency of Tregs in appendix lymphocyte infiltrate compared to colon and ileum lymphocyte infiltrate when analysed by IHC. The frequency of Tregs was also significantly higher in cancer appendix LP compared to cancer colon and ileum LP and a similar trend was seen in UC and CD mucosal tissues. Therefore it appears that the appendix generally has a higher frequency of Tregs compared to other intestinal sites. A higher frequency of Tregs is possibly required in the appendix as a higher proportion of IgG+ cells reside in it compared to colon and IgG has greater proinflammatory properties than IgA (Bjerke et al., 1986).

5.5.3 Circulating immunomodulatory cells in inflammatory bowel disease

5.5.3.1 Circulating T_{FH} in IBD

No difference in the frequency of cT_{FH} was observed between active UC and inactive UC patients and healthy controls but an increase in cT_{FH} was observed in active CD compared to healthy controls.

CXCR5 and PD-1 are also found on activated cells (Ansel et al., 1999, Agata et al., 1996). As well as the high frequency of circulating CXCR5+PD-1+ cells found in CD in this study, an increase in CXCR5+IL21+ cells has been observed in CD intestinal mucosa compared to UC and controls (Sarraf et al., 2010a). It is possible that this is due to an increase in activated cells rather than an increase in T_{FH} . This is especially relevant to inflamed intestine where the frequency of activated and effector cells is particularly high.

There is still some speculation as to whether circulating T_{FH} are related to T_{FH} found in GCs. It has been demonstrated that cT_{FH} are able to provide help to B cells and aid the generation of antibody secreting cells in response to antigen, however without exogenous stimulation, the amount of B cell help is far less than with GC-T_{FH} (Schaerli et al., 2000, Vinuesa and Cook, 2011, Morita et al., 2011). Therefore studying GC-T_{FH} maybe more disease relevant than studying cT_{FH}.

5.5.4 ***CXCL13 expression in IBD***

CXCL13 is important for the positioning of GC B and T cells and in the gut of CXCL13 deficient mice, small GC like clusters of T and B cells are able to form around the T cell zone but they lack the distinctive structure of peyer's patches in wild type mice (Ansel et al., 2000). However, aberrant expression of CXCL13 is associated with autoimmunity as elevated levels of CXCL13 have been observed in the serum of patients with SLE (Wong et al., 2010, Schiffer et al., 2009). As CXCL13 plays a role in the size and morphology of GCs and is linked to the generation of autoantibodies, the number of CXCL13+ cells within the gut of IBD patients and controls was assessed in this chapter.

Some differences in the number of CXCL13+ cells were observed in the lamina propria of UC tissues. However no difference in the number of CXCL13+ cells was observed between UC and control GCs. This is in agreement with observations made by Carlsen et al. (2002) where the expression of CXCL13 in UC GALT was similar to that in healthy GALT. This would suggest that altered expression of CXCL13 does not contribute to the small GC phenotype in UC or the production of disease associated autoantibodies.

CXCL13 is a secreted chemokine and therefore secreted CXCL13 is probably not visualized by IHC. Though there are equivalent numbers of CXCL13+ cells in UC and

control tissues, it is possible that the amount of CXCL13 they express is different; however, this has not yet been addressed. Carlsen et al. (2004) suggest that monocytes and macrophages significantly contribute to the levels of CXCL13 within inflamed UC gut tissue instead of FDC. It is possible that the source of CXCL13 could affect the morphology of the GC however there is no evidence to support this.

Interestingly, overall, equivalent numbers of CXCL13+ cells were observed in the lymphocytic infiltrate and GCs. Previous data has reported CXCL13+ cells in the mantle zone and high endothelial venules in healthy GALT to recruit lymphocytes into lymphoid tissue and retain them (Carlsen et al., 2002, Ebisuno et al., 2003). The CXCL13+ cells seen in the lymphocytic infiltrate maybe associated with these cells. However more research is needed in this area to establish the role of CXCL13 and CXCR5+ cells in the development and progression of UC.

5.5.5 *IL-17 expression in IBD*

In this study no significant difference in the number of IL-17+ cells was observed between UC, CD and cancer patients. However other studies have suggested that IL-17 is increased in IBD and a particularly high increase in IL-17+ cells is observed in both UC and CD mucosa (Fujino et al., 2003). Fujino et al. (2003) also observed a difference in the number of IL-17+ cells in inactive and active IBD patients. IL-17+ cells were more prevalent within active lesions in the intestine of patients with IBD (Fujino et al., 2003). The results in this chapter may differ from Fujino et al. (2003) results as a consequence of the type of tissue selected for this study. In figure 5.6 the IBD tissue was selected for being from part of the gut which was involved in the disease but only contained chronic inflammation. It maybe that an increase in IL-17+ is only found in acutely inflamed IBD tissue.

5.5.6 *Regulatory T cells in IBD*

In this study, no difference in the number of intestinal foxp3⁺ T cells was observed in IBD and control tissue by IHC. Previous studies have found an increase in foxp3 mRNA in both UC and CD intestinal tissue compared to control tissue (Wang et al., 2011b) and an increase in the number of Foxp3⁺ T cells within inflamed lesions compared to non-inflamed tissue (Maul et al., 2005). The apparent discrepancy between the data presented here and that of others, could be due to the nature of the tissue sampled. Maul et al. (2005) only found a significant difference in the number of foxp3⁺ T cells in severely inflamed tissue from IBD patients and even then the difference was not large. Interestingly, Maul et al. (2005) observed that there was a higher frequency of Tregs within the mucosa of diverticulitis inflammatory controls compared to IBD tissue and therefore suggested that there is in fact a deficiency in the number of Tregs in IBD. The equivalent frequency of Tregs within CD and UC intestinal mucosa and control cancer tissue is potentially consistent with the hypothesis proposed by others that CD and UC are deficient in Tregs, but this is difficult to gauge.

CD4⁺ T cells with the most suppressive ability are found in the CD127^{low}CD25^{high} gate when analysed by flow cytometry (Liu et al., 2006). In this study the activated and memory Treg subsets within the CD127^{low}CD25^{high} Treg population are reduced in active UC. Previous studies have also shown an overall reduction in the frequency of circulating Tregs in both active UC and CD compared to inactive disease (Maul et al., 2005). If the Treg population is depleted of activated Tregs which have the highest suppressive function, this could possibly lead to the loss of tolerance as seen in UC (Miyara et al., 2009).

The decrease in Treg frequency observed in the blood is believed to be due to the Tregs migrating to the site of inflammation in the gut mucosa (Saruta et al., 2007). Tregs increase in the inflamed tissue as a mechanism to control or resolve the immune

response. However, Maul et al. (2005) reported an increase in peripheral blood and mucosal Tregs in inflammatory diverticulitis controls, suggesting that there is actually a deficiency in the frequency of circulating Tregs in IBD. Supporting the theory that the decrease in Tregs in the peripheral blood of patients with active UC is not due to the Tregs migrating to the site of inflammation, no increase in Treg frequency was observed in CD or UC intestinal mucosa compared to cancer intestinal mucosa in this study.

Previous studies have shown that Tregs are functionally suppressive *in vitro* in IBD, therefore suggesting that numerical deficiencies in Tregs is more likely to contribute to the dsregulation of immune responses observed in IBD (Saruta et al., 2007). Analysis of functional properties of Tregs in IBD was not explored in this thesis due to time and financial constraints.

5.6 Conclusions

In this chapter the frequency and phenotype of T_{FH} and Treg were explored in context of IBD. The findings were:

- The relative density of T_{FH} is increased and GCs were smaller in UC appendix compared to control appendix.
- T_{FH} in UC appendix are mainly CD57+ and resemble the T_{FH} population in peripheral lymphoid tissues.
- The frequency of Tregs is higher in appendix than colon or ileum.
- Circulating memory and active Treg frequencies are decreased in active UC compared to inactive UC and healthy controls but no difference in Treg frequencies was observed in IBD tissue compared to control tissue.

6 Chapter 6: Overview

6.1 Definition of T_{FH} in tissue sections, cell suspensions and blood.

In this thesis T_{FH} have been analysed in tissue sections by immunohistochemistry, and in cells isolated from tissues and from blood by flow cytometry. In this thesis the ‘gold standard’ for T_{FH} definition was considered to be that T_{FH} should be located within a germinal centre. Therefore immunohistochemistry is the only method that can identify them unambiguously. When cell isolates were made it was necessary to depend on cell surface markers to identify T_{FH} . This is problematic in that some of the markers used are not T_{FH} specific and despite rigorous analysis and comparison between blood and tissues, an element of uncertainty remains. The subsets of T_{FH} identified in tissue sections were not apparent in the same way in cell suspension, and the first thought when trying to understand these differences is to challenge the content of the cell suspensions. In the future it would be interesting to pursue this more stringently and to combine further markers to resolve this issue. Only then could truly meaningful functional in vitro experiments be designed.

It would be useful in the future to be able to compare appendix and tonsil tissues in more detail in cell suspension. Unfortunately these tissues were not available in sufficient quantity during the time of this study, but many interesting questions remain. This is particularly important considering the intriguing profile of the appendix in UC. It would be interesting to know, for example, how T_{FH} from these different sites differ in their cytokine profile and their ability to support B cells in vitro.

6.2 Differences in the T_{FH} population in peripheral lymphoid tissue and GALT – implications for the pathogenesis of inflammatory bowel disease

In the first results chapter of this thesis, the density of T_{FH} in germinal centres from different microenvironments was compared. The ratio of T_{FH} to B cells was observed to be higher in the germinal centres of GALT compared to the germinal centres of peripheral lymphoid tissue. A higher density of T_{FH} has been linked with autoimmunity since this is thought to reduce the stringency of selection in the germinal centre microenvironment. The higher density of T_{FH} in GALT may be associated with the greater tendency for plasma cell precursors generated in GALT to go on to produce IgA with autoreactive and polyspecific components compared with systemic IgG responses.

In chapter 5 of this thesis it was observed that T_{FH} in the appendix in UC were present with higher density than either healthy GALT or other inflamed intestinal sites. This observation may be associated with the known local autoantibody production in UC and the inflammatory response, since selection pressure that would normally reduce the probability of autoantibody production would be lower.

Immunohistochemical analysis demonstrated that GCs in GALT contained a greater proportion of T_{FH} that lacked the antigen CD57 compared to those in peripheral immune responses. It is possible that $CD57^+$ T_{FH} develop from $CD57^-$ T_{FH} since CD57 is known to be associated with end stage differentiation.

The high density of T_{FH} in UC appendix was comprised almost exclusively of $CD57^+$ T_{FH} . T_{FH} in the UC are like peripheral lymphoid tissue in that the $CD57^-$ subset is relatively rare but are at a much high density than healthy peripheral lymphoid tissue or GALT. In addition, the GCs in the appendix in UC tend to be very small. The unusual composition of GCs in the appendix in UC may be related to disease

pathogenesis. Although the autoantibodies in UC are not known to be pathogenic, the immunological profile that generates a relatively high frequency of autoantibodies and a relative increase in IgG production may be relevant to the development of the inflammatory response. This may be a consequence of changes in the balance between immune modulatory T cells subsets as will be described below.

6.3 Two types of T cells with synergistic effects: regulatory T cells and T follicular helper cells

The immune system is constantly battling to maintain a balanced homeostatic equilibrium between activation and suppression. A successful immune response can be initiated by a pathogen and end with resolution of the infection and down regulation of the response. T_{FH} cells are involved in the generation of an immune response whereas T regs are involved in regulation and resolution and therefore these two cell types might be considered to be synergistic in their functions. In this thesis no evidence of a clonal relationship between T_{FH} and Treg was found, though it remains possible that T_{FH} and Tregs could function antagonistically and may be dependent in different ways on a common set of parameters.

A method for studying T cell plasticity in humans based on analysis of clones was refined in this thesis. Although no relationship between Treg and T_{FH} was found, clonality within the T_{FH} population was identified. Current methods for defining T cell plasticity involve culturing cells and observing switches from one profile to another. The advantage of the method developed in this thesis is that interrelatedness between T cell subsets could be investigated in vivo. It would be interesting in the future to use this method to determine whether T cell plasticity defined in vitro, for example between Th17 and Th1, is apparent in vivo. It would also be necessary to confirm T cell profiles

by functional study in vitro in such an experiment for comparison and confirmation of T cell lineage.

Overall, in this thesis, differences in T_{FH} have been described in different anatomical setting and in inflammatory disease. Future studies should be able to identify the functional relevance of these observations and their role in disease.

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